Low molecular weight plasma antioxidants in healthy individuals and head and neck squamous cell carcinoma patients

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Giving up is easy,

Continuing is hard,

The latter brings fruits,

The choice is ours…

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List of papers

Paper I Sakhi AK, Russnes KM, Smeland S, Blomhoff R, Gundersen TE. Simultaneous quantification of reduced and oxidized glutathione in plasma using a two-dimensional chromatographic system with parallel porous graphitized carbon columns coupled with fluorescence and coulometric electrochemical detection. J Chromatogr A. 2006 Feb 3;1104(1-2):179-89.

Paper II Sakhi AK, Blomhoff R, Gundersen TE. Simultaneous and trace determination of reduced and oxidized glutathione in minute plasma samples using dual mode fluorescence detection and column switching high performance liquid chromatography. J Chromatogr A. 2007 Feb 23;1142(2):178-84.

Paper III Svilaas A*, Sakhi AK*, Andersen LF*, Svilaas T, Strøm EC, Jacobs DR Jr, Ose L, Blomhoff R. Intakes of antioxidants in coffee, wine, and vegetables are correlated with plasma carotenoids in humans. J Nutr. 2004 Mar;134(3):562-7.

Paper IV Bohn SK*, Smeland S*, Sakhi AK*, Thoresen M, Russnes KM, Tausjø J, Svilass A, Svilaas T, Blomhoff R. Post-radiotherapy plasma total glutathione is associated to outcome in patients with head and neck squamous cell carcinoma. Cancer Lett. 2006 Jul 18;238(2):240-7.

Paper V Sakhi AK*, Bøhn SK*, Smeland S, Thoresen M, Smedshaug GB, Tausjø J, Svilaas A, Karlsen A, Russnes KM, Svilaas T, Blomhoff R. Post-radiotherapy plasma lutein, α-carotene and β-carotene are positively associated with survival in patients with head and neck squamous cell carcinoma. Manuscript.

Paper VI Sakhi AK*, Russnes KM*, Thoresen M, Karlsen A, Smeland S, Blomhoff R. Pre-radiotherapy plasma carotenoids and markers of oxidative stress are associated with survival in head and neck squamous cell carcinoma patients. Manuscript.

*These authors contributed equally.

Abbreviations

AGE	Advanced glycation end products
AIDS	Acquired immunodeficiency syndrome
ASAP	Antioxidant supplementation in atherosclerosis prevention
BHT	Butylated hydroxytoluene
BMI	Body mass index
BPDS	Bathophenanthroline disulphonate
COX	Cyclooxygenase
DHAA	Dehydroascorbic acid
DOPA	Dihydroxyphenylalanine
DMPO	Dimethyl-1-pyrroline N-oxide
D-ROMs	Derivatives of reactive oxygen species
ED	Electrochemical detector
ELISA	Enzyme-linked immunoabsorbent assay
ESCODD	European standards committee on oxidative DNA damage
ESR	Electron spin resonance
FAD	Flavin adenine dinucleotide
FFQ	Food frequency questionnaire
FLD	Fluorescence detector
FOX	Ferrous oxidation xylenol
GC-MS	Gas chromatography-mass spectrometry
GCS	gamma-glutamylcysteine synthetase
GJC	Gap-junctional communication
GGT	gamma-glutamyl transpeptidase
GPx	Glutathione peroxidase
GR	Glutathione reductase

Grx	Glutaredoxin
GS	GSH synthetase
GSH	Glutathione
GSSG	Glutathione disulphide / oxidized glutathione
GST	Glutathione-S-transferase
HDL	High-density lipoprotein
HER	1-hydroxyethyl radical
HNSCC	Head and neck squamous cell carcinoma
iNOS	Inducible nitric oxide synthase
IP	Isoprostane
LC-MS	Liquid chromatography-mass spectrometry
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoproteins
LOD	Limits of detection
MBB	Monobromobimane
MDA	Malondialdehyde
ORAC	Oxygen radical absorbance capacity
PBN	α-phenyl-tert-butylnitrone
Prx	Peroxiredoxins
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
SPT	Second primary tumours
Srx	Sulfiredoxin
SUVIMAX	Supplemantation en vitamins et mineraux antioxydants
TAC	Total antioxidant capacity

TBA	Thiobarbituric acid
TEAC	Trolox equivalence antioxidant capacity
TRAP	Total radical trapping antioxidant parameter
TR	Thioredoxin reductase
Trx	Thioredoxin
UV	Ultraviolet
WCRF	World cancer research fund
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
2,4-DNPH	2,4-dinitro hydrazine
8-iso-$PGF_{2\alpha}$	8-isoprostagladin $F_{2\alpha}$
8-oxo-dGua	8-oxo-7,8-dihydroguanine
8-oxo-dGuo	8-oxo-7,8-dihydro-2´-deoxyguanosine

1. Introduction

1.1 Oxidative stress

Oxygen was discovered by the Swedish scientist Carl Wilhelm Scheele in 1777. The quotation by Neil Young "The same thing that makes you live can kill you in the end" fits well with the functions of oxygen in aerobes. Oxygen is both essential and toxic to aerobes. The essentiality lies in the energy production via a class of chemical reactions called as reduction-oxidation or redox reactions. The toxicity is due to a number of free radicals and other reactive oxygen- and nitrogen species (ROS/RNS) produced during these normal cellular redox reactions as listed in Table 1.

Table 1 Examples of ROS and RNS

Free radicals	**Non-radicals**	
Superoxide, $O_2^{\bullet -}$	Hydrogenperoxide, H_2O_2	Dinitrogen tetraoxide, N_2O_4
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl	Peroxynitrite, $ONOO^-$
Peroxyl, $RO_2^{\bullet}$	Ozone, O_3	Peroxynitrous acid, ONOOH
Alloxyl, $RO^{\bullet}$	Nitronium ion, NO_2^+	Nitroxyl anion, NO^-
Hydroperoxyl, $HO_2^{\bullet}$	Singlet oxygen, $^1O_2$	Nitrosyl cation, NO^+
Nitric oxide, $NO^{\bullet}$		
Nitrogen dioxide, $NO_2^{\bullet}$	Dinitrogen trioxide, N_2O_3	

The main endogenous source for the production of ROS in eukaryotes is mitochondria via electron transport chain. The reduction of oxygen to water in mitochondria requires four electrons. Even under ideal conditions, there is leakage of electrons and 1-3% O_2 undergoes an incomplete singlet reduction producing reactive species especially superoxide anion ($O_2^{\bullet -}$) (1). Other potential endogenous sources are cytochrome P- 450 (phase 1 detoxification reactions), xanthine oxidase (purine degradation pathway), microsomes and peroxisomes (produce mainly hydrogen peroxide H_2O_2) , and inflammatory cell activation (1). Besides ROS, other reactive species like RNS are also produced. The most important source of nitrogen for the generation of RNS *in vivo* is nitric oxide ($NO^{\bullet}$) (2). It is known to be a product of the catalytic action of the nitric oxide synthase enzyme family on L-arginine (2). Recent evidence also suggests that it can be generated by reduction of nitrite, which can arise in the body by ingestion or from bacterial metabolism (2). Although nitric oxide ($NO^{\bullet}$) is not highly reactive with biological molecules it reacts rapidly with other oxygen radicals to generate highly damaging RNS such as peroxynitrite ($ONOO^-$), nitrogen dioxide radical ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3) (2) (Table 1). Enhanced formation of ROS/RNS also occurs as a consequence of diseases and from exogenous factors like tobacco smoke, environmental pollutants, drugs, ethanol and radiation (3). The reactivity and specificity of both ROS and RNS vary, hydroxyl ($OH^{\bullet}$) being the most reactive free radical with a half life of approximately 10^{-9} seconds (4).

The reactive species produced can both be useful and harmful. Among their useful functions, they are involved in pathogen defence, apoptosis, cell signalling pathways and regulation of transcription factors (5-7). .

The harmful effects of ROS/RNS are due to their non-enzymatic oxidation of various important cellular components like lipids, proteins and DNA. Lipid peroxidation occurs mostly in cellular membranes and low-density lipoproteins (LDL). The oxidation of lipids in plasma membranes alters their physical properties thereby changing their biological function. Oxidation of LDL is considered as one of the

factors contributing to atherosclerosis leading to cardiovascular disease (8). Protein oxidation leads to loss of function or premature degradation in several proteins thereby resulting in functional changes modulating cellular metabolism. Cataracts are thought due to photooxidation of lens proteins resulting in protein damage, accumulation, aggregation and precipitation in the lens (9). DNA oxidation can lead to gene mutation, and thus abnormal protein synthesis, alteration in gene expression, apoptosis and cell death. Oxidatively modified DNA may also play a role in carcinogenesis (10).

In order to counteract these highly reactive species, eukaryotic cells have developed a complex system of both enzymatic and non-enzymatic compounds, referred to as endogenous antioxidant defence. A critical balance is maintained between generation of ROS/RNS and the antioxidant defence. When there is an imbalance in the favour of ROS/RNS, either due to high production of ROS/RNS or due to impairment of antioxidant defence, these reactive species interfere with the normal function of ROS/RNS, and non-enzymatically oxidize and alter the structure and function of several cellular components such as lipids, proteins and DNA as described above. An accumulation of these oxidative damages will occur over time and result in oxidative stress. Oxidative stress is, thus, defined as “a condition that is characterized by accumulation of non-enzymatic oxidative damage to molecules that threaten the normal function of the cell or the organism” (3).

Compelling evidence has emerged in the last two decades demonstrating that oxidative stress is intimately involved in the pathophysiology of many types of diseases. Oxidative stress is now thought to make significant contribution to all inflammatory diseases (e.g. arthritis, vasculitis, glomerulonephritis, systemic lupus erythematosus, adult respiratory distress syndrome), ischemic diseases (heart disease, stroke, intestinal ischemia), cancer, hemochromatosis, acquired immunodeficiency syndrome (AIDS), emphysema, gastric ulcers, hypertension and preeclampsia, neurologic diseases (multiple sclerosis, Alzheimer’s disease, Parkinson disease, amyotrophic lateral sclerosis, muscular dystrophy), alcoholism, smoking-related

diseases, and many others as reviewed by McCord et al. (11). Additionally, a slow and steady accumulation of oxidative damage has also been considered as the major theory of aging (12).

1.2 Defence system against oxidative damage

As mentioned above, in order to minimize the generation and counterbalance the damaging effects of reactive species, eukaryotes have developed a comprehensive defence system. The defence system comprises of both enzymatic and non-enzymatic components, which works at different molecular aspects.

Preventive antioxidants suppress the formation and decrease the reactivity of reactive species. The enzymes involved in this process include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, peroxiredoxins and thioredoxin reductase. The non enzymatic components include polypeptides like thioredoxins, glutaredoxins and sulfiredoxins; metal binding proteins like transferrin, albumin; low molecular weight antioxidants like glutathione, uric acid; dietary antioxidants like vitamin E, ascorbic acid, carotenoids and polyphenols.

Another aspect in this defence is the repairing process and includes repair enzymes, which repair the damage and reconstitute membranes and DNA, for example lipase, DNA repair enzymes and transferases.

The defence system mentioned above could be called as “antioxidant defence system” since an antioxidant defined by Halliwell is any substance that delays, prevents or removes oxidative damage to a target molecule (13). A brief description of some of the endogenous and dietary antioxidants is given below.

1.2.1 Endogenous antioxidants

Antioxidant enzymes

The major enzymatic antioxidants present in eukaryotes are superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, peroxiredoxins and thioredoxin reductase.

Superoxide dismutase

Superoxide dismutase (SOD) was first discovered by McCord and Fridovich in 1969 (14). SOD catalyses the dismutation of two superoxide anions ($O_2^{\bullet -}$) to oxygen and less potent hydrogen peroxide (H_2O_2) in the following reaction:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

It is a pseudo first order reaction with rate constant of magnitude 10^9 $M^{-1}S^{-1}$ (15). Three major forms of SODs (cytosolic CuZnSOD, mitochondrial MnSOD and extracellular SOD) are present in animal cells based on their structure, localisation, inducibility and metal ion requirement. SOD containing other transition metals like FeSOD, Fe/MnSOD and NiSOD are present in some bacteria (13). All SODs destroys $O_2^{\bullet -}$ by successive oxidation and reduction of the transition metal ion at the active site. MnSOD is considered to be one of the most important antioxidant components of a cell and is largely located in mitochondria (15). MnSOD is a homotetrameric enzyme with Mn (III) at its active centre. CuZnSOD, a homodimeric protein, is located mainly in cytosol and requires both Cu (II) and Zn (II) at its active site. Cu (II) is essential for the enzymes catalytical activity, and Zn (II) imparts stability to the protein structure (15). Extracellular SOD, a tetrameric glycoprotein, also contains Cu (II) and Zn (II) and is found in the interstitial spaces of tissues and extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph and synovial fluid (16).

Reports involving SOD knock outs have revealed that MnSOD is essential for life and mice deficient in MnSOD gene die within 10-21 days after birth from

cardiomyopathy, metabolic acidosis and neurodegeneration (17;18). Mice lacking cytosolic CuZnSOD, however, appear normal when they are young, but with age they develop neurological damage, muscle wasting, hearing loss and cancers (especially liver cancer) at accelerated rates. They also have reproductive problems and show impaired vascular reactivity (13).

Catalase

Catalase was named by Loew in 1901 (14). The enzyme consists of four protein subunits, each containing a heme group [Fe(III)-protoporphyrin] bound to its active site (14). Catalase decomposes hydrogen peroxide (H_2O_2) into water and oxygen as shown in the following equation:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Catalase is ubiquitous to most aerobic cells. In animals, catalase is present in all major body organs and is especially concentrated in the liver and erythrocytes (14). At the subcellular level, catalase is found in peroxisomes (80%) and cytosol (20%) (14). Mice lacking catalase grow normally and show no obvious abnormalities, although their tissues showed a retarded rate in decomposing extracellular H_2O_2 (19). Additionally, these mice are more susceptible to trauma induced dysfunction in brain mitochondria (19).

Glutathione peroxidase

Glutathione peroxidase (GPx) was first described by Mills in 1957 (14). The enzyme reduces hydrogen peroxide (H_2O_2) to water with oxidation of GSH to glutathione disulphide (GSSG) in the following reaction:

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O$$

GPx are a family of selenium dependent and independent enzymes. They are widely distributed in animal tissues and are less common in plants or bacteria. The GPx enzymes are mostly specific for GSH as a hydrogen donor. They can however act on

other peroxides than hydrogen peroxide (H_2O_2). Four types of selenium dependent GPx exist, cytosolic/mitochondrial GPx (GPx1), cytosolic GPx (GPx2), extracellular GPx (GPx3 a glycoprotein) and phospholipid hydroperoxide GPx (GPx4) (16). GPx1 is present mainly in erythrocytes, kidney and liver; GPx2 in gastrointestinal tract; GPx3 in kidney and extracellular fluids like plasma, milk, seminal fluid, amniotic fluid, aqueous humor of the eye and lung lining fluid; Gpx4 is present ubiquitous but highest in renal epithelial cells and testis (13;16). GPx2 may serve to metabolize peroxides in ingested food lipids as well as generated during lipid peroxidation in the intestine itself (13). GPx4, located both in cytosol and the membrane fraction, has the unique ability to reduce not only hydrogen peroxide (H_2O_2) and synthetic organic peroxides but also fatty acid and cholesterol hydroperoxides that are still esterified (13). It is less specific for GSH as a reductant and can also reduce thymine hydroperoxide (product of free radical attack on thymine in DNA) (20) suggesting a possible in the repair of DNA damage. The GPx5, expressed specifically in rodent epididymis is selenium independent (13).

GPx1 knockout mice were healthy, fertile and showed normal phenotype (21). The animals, however, showed increased susceptibility particularly to ROS-mediated oxidative stress as compared to normal mice (22). Gpx4 homozygote knockout embryos die in utero by midgestation and were associated with a lack of normal structural compartmentalization (23). Gpx4 heterozygote displayed no morphological or behavioural abnormalities. The cell lines derived from heterozygotes were, however, sensitive to inducers of oxidative stress (23).

Glutathione reductase

Glutathione reductase (GR) was initially observed by Hopkins and Elliott in 1931 and later isolated by Mann in 1932 (14). GR converts GSSG back to GSH in the following reaction:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NAD^+$$

GRs contain two subunits, each with flavin adenine dinucleotide (FAD) at its active site. The enzyme is found in cytosol and mitochondria which matches GPx distribution (14).

Peroxiredoxins and thioredoxin reductases

Peroxiredoxins (Prx) are a family of nonseleno-peroxidases that reduce hydrogen peroxide (H_2O_2) and organic peroxides. They are homodimers and the redox reactions are dependent on cysteine at the active sites (13). Of the six mammalian members of this family, five (Prx1-Prx5) contain two conserved catalytic cysteines and utilize Trx as the reductant while Prx6 contains 1-cysteine and Trx is not involved in the reduction process (13;24). The Prx1, -2 and -6 occurs in cytosol whereas Prx3 only in mitochondria. The Prx4 form is found in the endoplasmic reticulum and extracellularly; and Prx5 in both mitochondria and peroxisomes (13).
Thioredoxin reductase (TR) is a FAD containing flavoenzyme, and along with polypepetide thioredoxin plays a key role in maintaining proteins in their reduced states (15). These enzymes contain selenium (as selenocysteine) and show similarities to GR (13)

Non-enzymatic antioxidants

The non-enzymatic group includes low molecular weight antioxidants, polypeptides and metal binding proteins.

Low-molecular weight antioxidants

Several low molecular weight antioxidants are synthesized *in vivo* like GSH, lipoic acid, uric acid, taurine, bilrubin, α-keto acids, melatonin, coenzyme Q, histidine-containing dipeptides, melanins, polyamines and plasmalogens. Among these antioxidants, GSH is one of the major cellular antioxidant.

Glutathione

Glutathione (GSH) is one of the most abundant cellular antioxidant, present in millimolar concentrations in most prokaryotic and in all eukaryotic cells, providing

protection against reactive species. The antioxidant activity comes from the free thiol group of GSH, which is easily oxidized, non-enzymatically by electrophiles and other oxidants. GSH also serves critical roles in detoxification of electrophiles and oxidants through enzymatic reactions with GPx and conjugation reactions catalyzed by glutathione-S transferase (GST) (25). Additionally, GSH has a predominant role in regulation of cellular and subcellular redox state, for example through reactions with glutaredoxin and protein disulfide isomerases to organize a proper tertiary structure of proteins through thiol-disulfide exchange (26). Agents altering GSH concentration have shown to affect transcription of detoxification enzymes, cell proliferation and apoptosis (26-29). Other vital functions of GSH in animals have been reviewed thoroughly (25;26;30).

The level of total GSH (i.e. sum of all forms of GSH) in human tissues normally ranges from about 1 to 10 mM, being most concentrated in liver (around 10 mM), spleen, kidney and erythrocytes (13;25). The liver is the main site of GSH synthesis and a net supplier of circulatory GSH via an active export mechanism (31;32). The intracellular concentrations of GSH and GSSG also vary considerably. Most of cellular GSH (85-90%) is present in cytosol with the rest in various subcellular organelles (25).

When the free thiol of GSH is oxidized, different oxidized forms of GSH may form including GSSG, mixed disulfides with free cysteine (GSSC) and protein bound GSH (PSSG, glutathionylation). The GSH/GSSG ratio is >10 under normal physiological conditions (25). During severe oxidative stress and detoxification reactions involving GSH, the concentration of free GSH may decrease and the concentration of GSSG may increase in the affected cells. However, during mild stress increased GSH concentrations (due to increased GSH synthesis) are often observed (33). Thus, many types of oxidative stress increased production of GSH through upregulation of γ-glutamylcysteine synthetase (GCS) (34). This upregulation provides protection from more severe stress and may be a critical feature of preconditioning and tolerance.

GSH synthesis

The cellular GSH level is replenished either from i) de novo synthesis, ii) γ-glutamyl transpeptidase (GGT, also called γ-glutamyl transferase) dependent recycling of extracellular GSH or other amino acids or iii) GSSG by NADPH-dependent GR.

De novo synthesis of GSH

The major determinant of GSH synthesis is the availability of cysteine and the level of the rate-limiting enzyme GCS, also called glutamate cysteine ligase (GCL). GSH is synthesised in the cell cytosol by the sequential actions of GCS and GSH synthetase (GS) (35). GCS catalyzes formation of the dipeptide γ-glutamylcysteine (γ-GC) from glutamate and cysteine, while GS catalyzes formation of the tripeptide GSH from γ-GC and glycine.

Mammalian GCS is a heterodimer comprising a heavy subunit (GCSh, 73 kDa) and light subunit (GCSl, 28 kDa) polypeptide each encoded by separate genes (36;37). Catalytical activity and GSH feedback inhibition are properties of the heavy subunit, but association with the catalytically inactive light or regulatory subunit can significantly influence its enzymatic activity by promoting high affinity for glutamate and appropriate sensitivity to GSH feedback inhibition (37;38).

Formation of GSH through GGT dependent pathways

GSH contains a γ-peptide linkage between glutamate and cysteine (Figure 1).

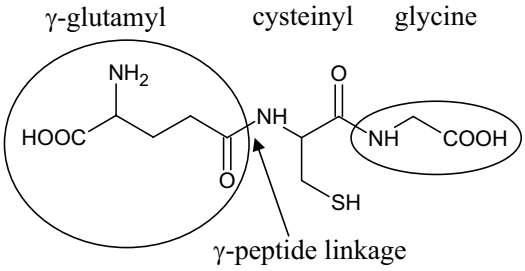


Figure 1 Structure of GSH showing γ-peptide linkage between amino acids glutamate and cysteine

The γ-peptide bond promotes stability, as it is resistant to degradation by cellular peptidases. Plasma membrane bound GGT may, however, cleave the γ-peptide bond of extracellular GSH, and thereby support intracellular synthesis of GSH (39). This

cleavage of GSH is important since GSH can not be taken up by most cells, in contrast to the cysteine, which is formed by the action of GGT and a dipeptidase. GGT additionally facilitate the extracellular formation of γ-GC (40). γ-GC may then be taken up by cells where it can be further metabolized to GSH. These GGT dependent pathways may help maintain the GSH level in the short term when GSH is rapidly utilized, but they are not sufficient to restore GSH levels in the long term when de novo synthesis of GSH is needed. Upregulation of plasma membrane bound GGT is often seen in oxidative stress, and free plasma GGT is a useful biomarker for oxidative stress (41).

Formation of GSH through glutathione reducatse

The intracellular GSH level is also influenced by the flavoenzyme GR and the family of selenium dependent GPx. GPx catalyzes the oxidation of GSH with the concomitant reduction of hydro- and lipid-peroxides, thereby resulting in the formation of GSSG. GR reduces GSSG to GSH through a NADPH-dependent reaction (33).

GSH is in a constant state of turnover; its half life has been estimated as 5 hours in rat liver, 5 days in human erythrocytes and few minutes in human plasma (42-44).

Numerous studies have demonstrated that GSH is an essential part of the antioxidant defence in all eukaryotic organisms. For example, experimental deletions of genes responsible for GSH synthesis in animals are lethal (especially in GCSh homozygous knock outs) and increases the vulnerability for oxidative stress, while overexpression of the same genes leads to enhanced defence against oxidative stress and extends life span (45-47). Furthermore, inborn genetic errors of such genes in humans are known to cause a wide spectra of GSH deficiency symptoms as reviewed by Townsend et al. and Ristoff et al. (30;48).

Uric acid

Uric acid is produced from hypoxanthine and xanthine by xanthine oxidase (XO) and xanthine dehydrogenase (XDH) enzymes (49). In most species, urate oxidase

converts it further to allantoin and then gloxylate plus urea. Urate oxidase is absent in humans causing an accumulation of uric acid in plasma (49). The concentration of uric acid in human plasma is 0.05-0.9 mM (49). At physiological pH, it is present as urate ion since pKa of uric acid is around 5.4. Ames et al. (50) suggested that urate is a powerful scavenger of ROS *in vitro*, proposing that it functions as a biological antioxidant. Urate reacts with several reactive species like hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), ozone (O_3), nitrogen dioxide radical ($NO_2^{\bullet}$), peroxynitrite ($ONOO^-$) and oxo-heme oxidants produced by peroxide reaction with haemoglobin (13;50). Urate binds metal ions like Cu and Fe making them poorly reactive in catalysing free-radical reactions (51;52). These reactions generates urate radical and the unpaired electron is localised over the purine ring, giving a resonance stabilized radical that does not react with oxygen to form peroxyl radical ($RO_2^{\bullet}$) (13). The role of urate as an antioxidant is, however, controversial. Recently, large clinical studies have shown a high risk of cancer and cardiovascular mortality with high levels of serum uric acid (53-56).

Polypeptides –Thioredoxins, glutaredoxins and sulfiredoxins

Thioredoxins are polypeptides of relative molecular mass about 12000, found in both prokaryotes and eukaryotes (13). Three different variants of human Trx have been described: cytosolic Trx1, mitochondrial Trx2 and SpTrx which is highly expressed in spermatozoa (57). Both Trx1 and Trx2 are ubiquitously present in humans (58). Reduced thioredoxins contain two –SH groups in a conserved -Cys-Gly-Pro-Cys- active site that form a disulphide in oxidized thioredoxin (57). Trx are involved in a variety of functions including reduction of ribonucleotide reductase and several transcription factors such as p53, nuclear factor-κB (NF-κB), activator protein-1 (AP-1), thereby playing a protective role against oxidative stress (57). They bind to target proteins and via intermediate formation of a mixed disulphide, reduce the protein disulphide bridge while oxidising themselves (13). Oxidized Trx are reduced *in vivo* in animals by TR. **Glutaredoxins** are thiol-disulphide oxidoreductases requiring GSH for their catalytical functions (15). Grx are present in most living organisms and catalyse the reduction of protein disulphide to their

respective sulphydryls by donating reducing equivalents to the oxidized proteins (15). The oxidized Grx gets reduced by GSH, which is oxidized to GSSG. **Sulfiredoxins** are mainly present in eukaryotes and are involved in reduction of cysteine-sulphinic acid in the 2-cysteine Prx enzymes (59). They are also involved in deglutathionylation of proteins (60).

Metal binding proteins

Although metals like Cu, Zn and Fe are essential in almost all eukaryotes for the synthesis of various proteins involved in respiration, O_2 transport and antioxidant defence. However, these metals also act as prooxidants and convert less reactive to more reactive species, for example Fe (II) and Cu (I) catalyses the formation of more reactive hydroxyl radicals ($OH^{\bullet}$) from less reactive hydrogen peroxide (H_2O_2) (13).

Various metal binding proteins are present in animal cells that bind metal ions thereby making them non-toxic. **Metallothioneins** are metal binding low molecular weight thiol proteins that are involved in the sequestration and distribution of metal ions especially Cu and Zn, removal of heavy metal ions from extracellular space (61). These proteins are rich in sulphur containing 22-33% cysteine thereby contributing significantly to total cellular protein thiol (13). The high –SH content also make them excellent scavengers of peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\bullet}$) (13). Other proteins like transferrin, ferritin and lactoferrin bind Fe (13). **Caeruloplamin** binds Cu and also exhibits ferroxidase activity; it oxidizes Fe (II) to Fe (III) and can facilitate iron loading on to transferrin and possibly ferritin (13). It also exhibits peroxidase activity in lung lining fluids (62). Fe can still act as prooxidant in some bound forms like haem and haem proteins. Plasma, however, contains haemoglobin-binding haptoglobins, as well as a haem-binding protein (haemopexin). The binding of haemoglobin and haem to haptoglobin and haemopexin respectively, decreases their effectiveness in stimulating lipid peroxidation (13).

Malfunctioning of the metal-binding proteins increases the concentrations of free metal ions making cells susceptible to oxidative damage as reviewed by Halliwell et al. (13).

Albumin

Albumin is a small, highly soluble plasma protein at a concentration of about 40 mg/ml (63). It contains an exposed –SH group at position 34 and contributes up to 500 μM to total plasma thiols (13). Albumin has multiple roles including being an important extracellular antioxidant. Among its antioxidant roles, it binds Cu tightly and Fe weakly (13). It also binds haem thereby protecting lipoproteins against haem-dependent oxidation (13). Albumin-SH reacts quickly with peroxynitrite ($ONOO^-$), nitrogen dioxide radical ($NO_2^\bullet$), hypochlorous acid (HOCl), peroxyl ($RO_2^\bullet$)- and alkoxyl ($RO^\bullet$)- radicals, and slowly with hydrogen peroxide (H_2O_2) (13).

1.2.2 Dietary antioxidants

Epidemiological studies show that a higher intake of fruits and vegetables decreases the risk of developing diseases like cardiovascular disease, stroke and certain types of cancer (64-68). The mechanisms behind this beneficial effect of fruits and vegetables are not fully understood. But we know that plants synthesize different phytochemicals that protect them against oxidative damage during photosynthesis and other abiotic stresses like drought, heat, cold and frost, effects of radiation levels, shade, altitude, soil nutrient and pollution (69). Since these phytochemicals are protective against oxidative damage, they are called as plant-antioxidants or dietary antioxidants. These antioxidants vary in their structure, physical and chemical properties and thus divided into various groups. Most commonly known groups are carotenoids, tocopherols, vitamin C and polyphenols.

Plasma levels of some of these dietary antioxidants in humans are also inversely related to prevalence of some of these diseases and mortality (70-79). These diseases have oxidative damage and oxidative stress as underlying mechanisms in their pathogenesis. It was, thus, hypothesized that (a) these dietary antioxidants may play a

role in the prevention of human disease and (b) their mode of action is through their antioxidant properties, thereby reducing oxidative damage and development and progression of diseases.

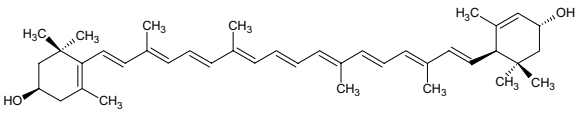
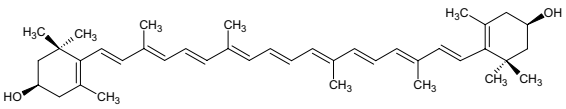
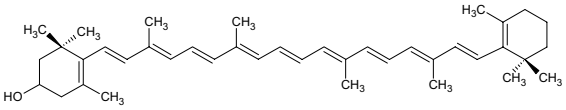
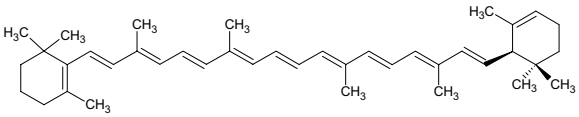
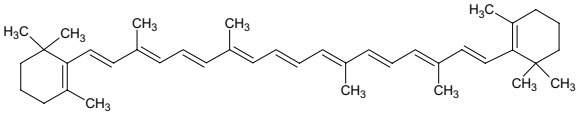
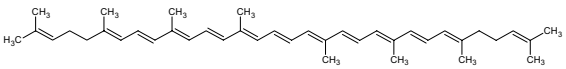
The description of some of these dietary antioxidants and mode of their action both *in vitro* and *in vivo* systems is described as follows:

Carotenoids

Carotenoids are lipid soluble pigments (usually yellow, red or orange) that are widespread in plants. They are also present in some microorganisms and animals (snails, goldfish, salmon, bird plumage and lobsters) (13). Carotenoids belong to the tetraterpenes family, basic structure being a symmetrical tetraterpene skeleton containing eight isoprene units. Thus, all carotenoids possess a long conjugated chain of double bond and a near bilateral symmetry around the central bond (80). Different carotenoids are derived by modifications in the basic structure by hydrogenation, dehydrogenation, cyclization or oxidation (80). The class of carotenoids containing only carbon and hydrogen atoms are called as carotenes (β-carotene, α-carotene and lycopene) whereas xanthophylls carry at least one oxygen atom (lutein, zeaxanthin). Table 2 presents some of the major carotenoids. Due to the conjugate double bonds, carotenoids could exist as cis- or trans- geometric isomers. Trans-isomers are more common in nature presumably due to more stability of the long polyunsaturated chain in the linear, extended trans-form (80).

Fruits and vegetables constitute the major sources of carotenoids in human diet (Table 2). More than 600 different carotenoids have already been identified in nature. However, 40 carotenoids are present in a typical human diet and 20 carotenoids have been identified in human blood and tissues (81). Major dietary carotenoids include the hydrocarbons, β-carotene, α-carotene and lycopene and the xanthophylls, or oxygen-containing carotenoids, β-crytoxanthin, lutein and zeaxanthin.

Table 2 Structure and dietary sources of some major carotenoids

Carotenoids	Dietary sources
Lutein 	spinach, kale, broccoli, brussel sprouts
Zeaxanthin 	egg yolks, maize, spinach
β-cryptoxanthin 	citrus fruits, avocado, papaya, pepper
α-carotene 	Carrots, pumpkin, maize
β-carotene 	Carrots, spinach, parsley
Lycopene 	Tomato and its products, water melon, guava

The tissue and plasma levels of carotenoids vary with diet and bioavailability. The bioavailability depends upon how the food is processed and the type of food matrix in which carotenoids are located. Processing, such as mechanical homogenisation or heat treatment enhances the bioavailability (82). The ingestion of fat along with carotenoids also increases their intestinal absorption (82).

Carotenoids are transported by lipoproteins in human plasma. The distribution of carotenoids among lipoproteins is, however, dependent upon their physical properties: β-carotene, α-carotene and lycopene (hydrocarbons) tend to be localised predominantly in the low density lipoproteins (LDL), lutein and zeaxanthin (dihydroxy) are more localised in high density lipoprotein (HDL) whereas β-cryptoxanthin (hydroxy) is equally distributed between LDL and HDL (83). Additionally, hydrocarbons (lipophilic) are located in the core of lipoproteins whereas xanthophylls (polar) are located on the surface. Plasma carotenoids were measured in 3043 individuals from 16 different regions in Europe and a huge variation in plasma levels due to region of residence was observed (84). After region, BMI was the second most important predictor followed by smoking status, gender, season and alcohol intake for the variation in plasma levels of carotenoids (84).

The antioxidant action of carotenoids in cell free experiments involves the ability of these pigments to physically quench or inactivate singlet oxygen ($^1O_2$). In doing so ground state oxygen and triplet state of carotenoid is generated. The triplet state of carotenoid returns to ground state by dissipating its energy through rotational and vibrational interactions with the solvent system (85). The efficacy of carotenoids for physical quenching depends upon the number of conjugated bonds that determines their lowest triplet energy state, presence of the functional groups in the molecule and the type of solvent used (86;87). Among different carotenoids, lycopene showed the highest quenching ability of singlet oxygen ($^1O_2$) (86). Carotenoids are also shown to react with other free radicals including chain-propagating peroxyl radicals ($RO_2^{\bullet}$) which are generated in the process of lipid peroxidation (88). The reaction with free radicals is shown to act by three pathways: radical addition, electron transfer or

hydrogen abstraction depending upon the physical property of the carotenoids, type of reactive species and the environment (polar or non-polar) employed to study these reactions (87;88).

The *in vitro* experiments have shown that added carotenoids prevent LDL oxidation, decrease DNA oxidation in lymphocytes (85). The *in vivo* studies done with animals have demonstrated an antioxidant action of carotenoid as reviewed by Krinsky et al (85). However, most experimental animals are very poor absorbers of carotenoids and only large pharmacological doses of carotenoids permit their absorption in these animals.

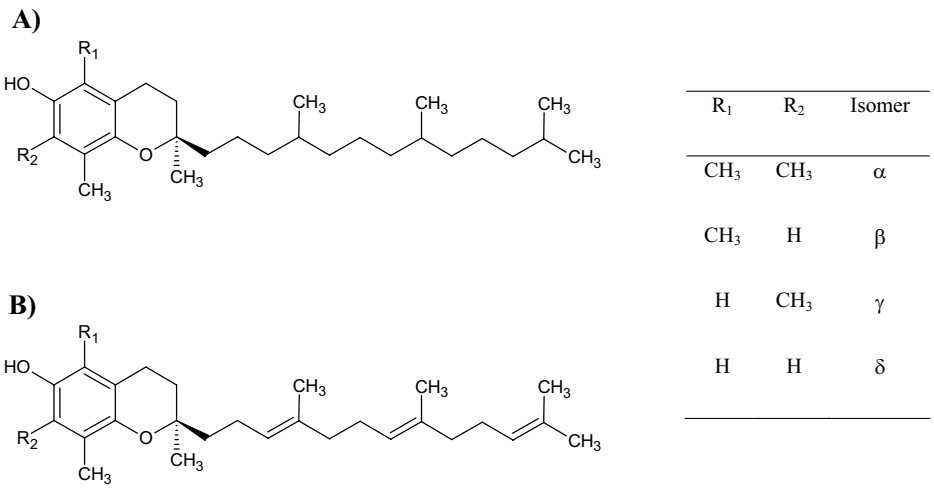
The best established role of carotenoids in humans is as a precursor of the fat-soluble vitamin A. About 50 carotenoids (not including lycopene) can generate vitamin A, the important ones being β-carotene, α-carotene and β-cryptoxanthin (89;90). Additionally, the recent report by World Cancer Research Fund (WCRF) (91) concluded that foods containing carotenoids probably protect against mouth, pharynx, larynx and lung cancer; while foods containing β-carotene and lycopene probably protect against oesophagus and prostate cancer respectively. The *in vivo* protective action of supplemental β-carotene has been questioned (92).

Carotenoids can also act as prooxidants in cell models, but the prooxidant action is observed under certain circumstances namely high oxygen tension, high carotenoid concentration, unbalanced intracellular redox status (93). However, there is no evidence to support the hypothesis that dietary carotenoids may act as prooxidants within a biological system i.e. at physiological relevant partial pressure of oxygen.

Carotenoids also exhibit other non-antioxidant functions like immunomodulatory actions, induction of gap-junctional communication (GJC). During carcinogenesis, GJC is lost and this loss may be important for malignant transformation, and its restoration may reverse malignant processes (94). Carotenoids stimulate GJC in a differential and dose-dependent manner; however the underlying mechanisms are not yet understood (94).

Vitamin E

Vitamin E was discovered by Evans and Bishop in 1922 and is a generic name for eight different isoforms with biological activity that have been isolated from plant sources: α-, β-, γ- δ- tocopherol and α-, β- γ- and δ- tocotrienol (Fig 1) (95). Tocopherols differ from tocotrienols only in their aliphatic chain. Tocopherols have a phytyl side chain attached to their chromanol nucleus, whereas the tail of tocotrienols is unsaturated and forms an isoprenoid chain (95). The various isoforms differ in their methyl substituents on the chromanol nucleus as shown in Figure 2. Each tocopherol has 3 asymmetric carbon atoms giving 8 optical isomers. Humans absorb all forms of vitamin E, but the body maintains only RRR-α-tocopherol, formerly called as d-α-tocopherol (96). Dietary sources of Vitamin E are vegetable oils, nuts (especially almonds and hazelnuts), wheat-germ and grains (13).



R_1	R_2	Isomer
CH_3	CH_3	α
CH_3	H	β
H	CH_3	γ
H	H	δ

Figure 2 Different isomers of vitamin E- tocopherols (A) and tocotrienols (B)

Vitamin E is a fat-soluble vitamin located in the lipophilic compartment of membranes and lipoproteins. This vitamin was discovered as a micronutrient that was essential for the reproduction in female rats. In humans, vitamin E deficiency

primarily causes neurologic dysfunctions, but the underlying molecular mechanisms are unclear (96). Vitamin E is also considered to be one of the most important lipid soluble antioxidants.

As an antioxidant, vitamin E is an important inhibitor of lipid peroxidation as it scavenges chain propagating lipid peroxyl radicals ($RO_2^{\bullet}$) much faster than these radicals can react with adjacent fatty acid side chains or with membrane proteins. The rate constant of this reaction *in vitro* is 10^4 $M^{-1}s^{-1}$, three orders of magnitude higher than reaction of peroxyl radicals ($RO_2^{\bullet}$) with lipids (50 $M^{-1}s^{-1}$) (97). The importance of this function is to maintain the integrity of long-chain polyunsaturated fatty acids in the membranes of cells and thus maintain their bioactivity (98). During its action as a chain breaking antioxidant, α-tocopherol forms a radical, which is resonance stabilized due to its chromanol nucleus. This radical is capable of reacting with another peroxyl radical ($RO_2^{\bullet}$) to give non-radical products and thus one molecule of α-tocopherol is in principle capable of terminating two peroxidation chains (13). The tocopherol radical can also be reduced back to tocopherol through hydrophilic antioxidant ascorbic acid as shown by Constantinescu et al. (99) and Sharma et al. (100).

The non-antioxidant aspect of vitamin E is also studied which includes its antiatherosclerotic and anticarcinogenic properties through modulation of cell signalling, transcriptional regulation and induction of apoptosis. There is still no agreement if vitamin E acts through its antioxidative or non-antioxidative properties at physiological levels in humans as reviewed by Traber et al. (98) and Azzi et al. (101).

Finally, a review by Brigelius-Flohe et al. (96) analysing the vitamin E function and metabolism concludes that the functions of vitamin E are underestimated if only its antioxidant properties are considered and further research is required to study the essentiality of this vitamin for humans.

Ascorbic acid

Ascorbic acid, also called as vitamin C was isolated by Szent-Györgyi in 1928. Ascorbic acid is a water soluble five-membered lactone containing two ionisable –OH groups, with pKa1 and pKa2 as 4.25 and 11.8 respectively (Figure 2) (102). Its acidity (pK_{a1} = 4.25) exceeds of weak carboxylic acid such as acetic acid (pKa = 4.75) due to the resonance stabilization of the monoanion form (102). Thus, at physiologic pH the monoanion ion form is favoured contributing more than 99% (102). Plants and some animals can synthesize ascorbate from glucose but humans, other primates, guinea pigs, some fish and fruit bats do not have the enzyme required for the terminal step (gulonolactone oxidase) and need ascorbate in the diet (13). Dietary sources of this vitamin include citrus fruits, guava, berries, mango, broccoli and peppers (13).

Figure 3 Structures of ascorbic acid (A) and dehydroascorbic acid (B)

Deficiency of ascorbic acid causes scurvy with symptoms of spongy, bleeding gums leading to tooth loss, poor wound healing and swollen, weakened limbs (103). The molecular mechanisms of the antiscorbutic effect of ascorbic acid are largely, but not completely understood (103). It is a cofactor of at least eight enzymes involved in collagen biosynthesis. Collagen synthesized in the absence of ascorbate is insufficiently hydroxylated and does not form fibres properly, giving rise to poor wound healing and fragility of blood vessels (13). The current recommended dietary allowance (RDA) for ascorbic acid is 60 mg/day for healthy, non-smoking adult (103).

Besides being an antiscorbutic, ascorbic acid is an excellent water-soluble reducing agent/antioxidant in biological fluids. It readily scavenges various ROS and RNS, such as superoxide anions ($O_2^{\bullet-}$), hydroperoxyl radical ($HO_2^{\bullet}$) , peroxyl radical ($RO_2^{\bullet}$), singlet oxygen ($^1O_2$), ozone (O_3), peroxynitrite ($ONOO^-$), nitrogen dioxide radical ($NO_2^{\bullet}$) and hypochlorous acid (HOCl), thereby protecting lipids, proteins and DNA from oxidative damage (13). Ascorbic acid also regenerates lipophilic tocopherol from its radical, produced by scavenging of lipid-soluble radicals (99;100). The very low reduction potential of ascorbate and stability and low reactivity of the ascorbyl radical formed after scavenging reactive species makes it an ideal antioxidant and is also called as the “at the bottom of the pecking order” or “terminal water-soluble small molecule antioxidant” (104) . The ascorbyl radical either disproportionates to ascorbate and dehydroascorbic acid (DHAA) (Figure 3) or is reduced back to ascorbate by NADH-dependent semihydroascorbate reductase. DHAA is unstable at physiological pH, with a half life of about 6 min (105) and is rapidly and irreversibly hydrolysed to 2,3-diketogulonic acid and leads to the depletion of this vitamin. DHAA can, however be reduced back to ascorbate enzymatically by GSH-dependent DHAA reductase (106). Other proteins like glutaredoxin, thioredoxin reductase, protein dislulphide isomerase can also act as DHAA reductases (106;107).

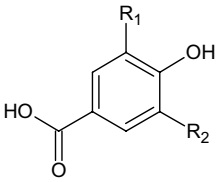
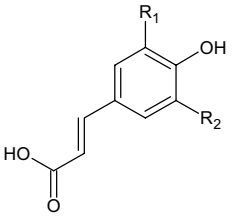
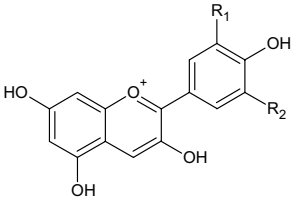
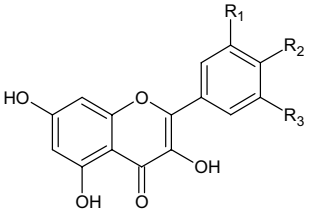
Ascorbate can also act as a prooxidant *in vitro*. Being a powerful reducing agent, it reduces catalytic metal ions Fe (III) and Cu (II) to Fe (II) and Cu (I) respectively. These reduced metal ions are required in the Fenton reaction for the production of hydroxyl radicals ($OH^{\bullet}$). The prooxidant activity depends upon the concentration and form of metal ions, and a high concentration of free metal ions are required for this effect (102;108). The *in vivo* evidence for metal ion dependent prooxidant action is, however, sparse (108). Nevertheless, for patients suffering from iron-overload supplemental ascorbic acid could be detrimental and high doses are not recommended (109).

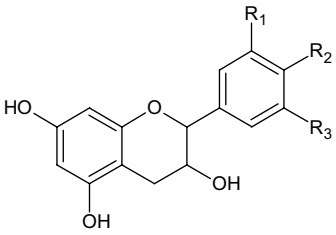
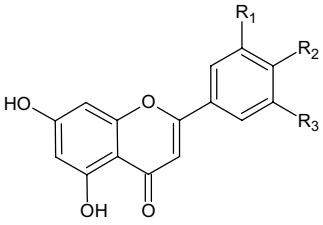
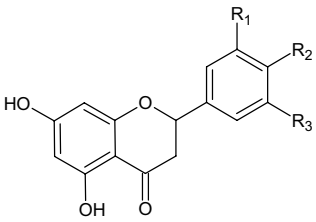
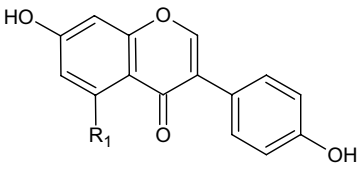
As common for other dietary antioxidants like vitamin E and carotenoids, roles of ascorbic acid other than being an antioxidant are explored. Recent findings on the specific requirement of ascorbate for the activity of several 2-oxoacid-dependent dioxygenases involved in cell signalling and the activation of transcription factors opens new fascinating area for future research (109;110).

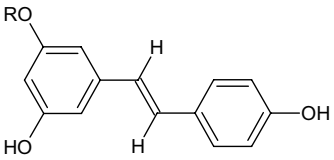
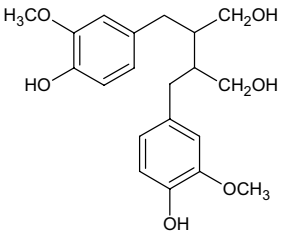
Polyphenols

Polyphenols represent a wide variety of compounds and are characterised by having more than two –OH groups. The different classes of polyphenols are hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, and lignans (Table 3) (111-113). The total dietary intake is about 1 g/day and is much higher than other known antioxidants, about 10 times higher than ascorbic acid and 100 times higher than vitamin E and carotenoids (114). Bioavailability of polyphenols differs greatly; the absorption is accompanied by extensive conjugation and metabolism such that the forms appearing in the blood are usually different from the forms found in food as reviewed by Scalbert et al. (114) and Manach et al. (111). The plasma concentrations of total metabolites ranged from 0-4 μmol/L with an intake of 50 mg aglycone equivalents (111).

Table 3 Structures and dietary sources of different polyphenols.

Polyphenols	Examples	Food sources
Hydroxybenzoic acid	Gallic acid	Tea
		
Hydroxycinnamic acid	Caffeic acid	Coffee, white grapes
	Chlorogenic acid	Apples, cherries, pears
	p-coumaric acid	White grapes, tomatoes, spinach
Anthocyanins	Cyanidin	Cherries, raspberry
	Malvidin	Strawberries, blackberries, grapes
Flavonols	Quercetin	Onions, lettuce
	Myricetin	Cranberry, grapes
	Kaempferol	Endive, leek, broccoli

Polyphenols	Examples	Food sources
Flavanoles	Epicatechin	Green tea
	Gallocatechin	Apple, cocoa
Flavones	Apigenin	Parsley
	Luteolin	Celery
	Diosmetin	Sweet pepper
Flavanones	Naringenin	Citrus foods
	Hesperedin	Prunes
Isoflavones	Genistin	Soybeans
	Daidzein	Legumes

Polyphenols	Examples	Food sources
Stilbenes 	Resveratrol	Red grapes
Lignans 	secoisolariciresinol	linseed

In vitro, most polyphenols exert antioxidant effects, inhibiting lipid peroxidation by acting as chain-breaking peroxyl radical ($RO_2^{\bullet}$) scavengers. In addition, phenols often scavenge other reactive species such as hydroxyl radicals ($OH^{\bullet}$), nitrogen dioxide radical ($NO_2^{\bullet}$), dintrogen trioxide (N_2O_3), peroxynitrous acid (ONOOH) and hypochlorous acid (HOCl) (13). Some can react with superoxide anions ($O_2^{\bullet-}$), mostly the di- and polyphenols. The number of phenolic groups and their relative positions are key determinants of antioxidant activity as the phenolic groups can accept an electron to form relatively stable phenoxyl radicals. Some polyphenols with adjacent –OH groups can also act as metal chelators and can bind transition metal ions especially Fe and Cu often in forms poorly active in promoting free-radical reactions (13). This chelating ability of polyphenols can interfere with uptake of metals from the diet.

The *in vitro* effects of polyphenols on the DNA oxidation are both harmful and beneficial (115). Polyphenols can have harmful effects in the presence of transition metal ions such as Cu (II) and Fe (II). Polyphenols reduce these metal ions and the

reduced forms catalyze the formation of free radicals like hydroxyl ($OH^{\bullet}$) which cause DNA oxidation. Such breakage of DNA has been considered both beneficial (cytotoxic and apoptotic effects on tumor cells) and toxic (mutagenic effects on normal cells) (115).

Some, but not all, in vivo studies in animals (polyphenol compounds) and humans (polyphenol-rich foods or beverages) have demonstrated their protective effect against DNA damage and reduced susceptibility to LDL oxidation (115;116). Besides being antioxidants, they also exert antithrombotic effects (115).

Intervention with supplemental antioxidants

Foods containing phytochemicals such as carotenoids, ascorbic acid, vitamin E and quercetin are protective against some cancers (91). However, the majority of studies find that antioxidant supplements (β-carotene, α-tocopherol and ascorbic acid) do not decrease the risk of oxidative stress related diseases and mortality. A meta-analysis by Bjelakovic et al. (117) and a recent report from WCRF (91) have shown that antioxidant supplements may increase total mortality and risk of developing lung cancer, respectively.

Possible explanations for these apparent conflicting results between dietary and supplemental antioxidants could be: The beneficial effect could be due to multiple antioxidants present together and working simultaneously in a network (as in fruits and vegetables) rather than single antioxidants. Thus, it is suggested that low doses of many antioxidants may contribute to a positive antioxidant defence network, while large doses of one or a few antioxidants as typically used in supplements may have prooxidant effects. For example in Supplemantation en Vitamines et Mineraux AntioXydants (SU.VI.MAX) study (118) and Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study (119), the intervention group received low doses of α-tocopherol, ascorbic acid , β-carotene and a protective effect against cancer and atherosclerosis was observed in men.

1.3 Assessment of antioxidant and oxidative stress biomarkers in clinical studies

An imbalance between reactive species and defence system causes oxidative stress. The assessment of this oxidative stress can be approached by measuring reactive species produced either directly or the damage produced by them i.e., oxidative damage. The decrease in the antioxidant defence due to their utilization during oxidative stress is another approach for this assessment.

1.3.1 Measuring reactive species

The measurement of reactive species can be done by two approaches:

- Trapping of these reactive species and measuring the trapped species by a technique called as electron spin resonance (ESR) or electron paramagnetic resonance (EPR).
- Measuring the oxidative damage done by reactive species, i.e., the amount of oxidative damage.

Trapping of reactive species

ESR is a spectroscopic technique that detects unpaired electrons and is thus specific for free radicals. The free radicals like superoxide anion ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) are, however, highly reactive with reaction time of microseconds to nanoseconds and this technique is not sensitive enough to detect them directly in living systems. These radicals are trapped by a trap molecule, forming more stable radicals that accumulate to a level permitting their detection by ESR (120). The ideal trap should react rapidly and specifically with the radical one wishes to detect, to produce a product that is chemically stable, not metabolized by living systems, and has a unique ESR spectrum (13). A wide range of traps are available for the use in animals and cell cultures (13;121). Examples of some of these trap molecules include α-phenyl-tert-butylnitrone (PBN), 5,5-dimethyl-1-pyrroline N-oxide (DMPO).

Whole-body ESR techniques are being used on animals (122) but currently not on humans due to lack of human safety data for new probes. For humans, traps have been used ex vivo on body fluids and tissues like biopsies, blood and other tissue samples (120). Although the traps are added in ex vivo samples, it is impossible to detect free radicals like hydroxyl ($OH^{\bullet}$) with a reaction time of nanoseconds. ESR of ex vivo samples probably detects secondary radicals resulting from the reaction of these reactive species with biomolecules. Some of the examples are lipid derived radicals such as alkoxyl ($RO^{\bullet}$)- and peroxyl ($RO_2^{\bullet}$)- radicals (120). Ascorbic acid also reacts with a large number of reactive species generating radical semidehydroascorbate radical (123). This radical can be detected by ESR and have been used as an indication of free radical production in organs, plasma and skin (100;124;125). Among the *in vivo* ESR in humans, aromatic free radical traps like salicylate and phenylalanine have been used to detect hydroxyl radicals ($OH^{\bullet}$) with some success as reviewed by Halliwell et al. (120).

The potential limitations in the use of spin trapping technique are efficiency of radical trapping, selectivity and availability of spin traps, the limited stability of spin adducts, possible formation of artifactual spin adducts and under estimation due to removal of products giving ESR signal (126). For example, when DMPO is used to trap hydroxyl radicals ($OH^{\bullet}$), any ascorbate present can directly reduce the adduct between DMPO and hydroxyl radicals (DMPO-$OH^{\bullet}$) to an ESR-silent species. With the development of new and better spin traps going on, ESR can be a very effective and powerful technique in studying processes involving free radicals.

Measuring oxidative damage

An alternative to trapping is the method in which the reactive species are not measured themselves but the damage caused by them. When reactive species attack biomolecules, the oxidized products formed can be used as biomarkers to measure this damage. Most human studies focus on the measurement of oxidative damage rather than the total reactive species generated because of limited applicability to humans of the latter technique. Moreover, it is the damage that matters rather than the

total amount of free radicals generated. Criteria for a valid biomarker as described by Halliwell et al. (120) and Griffiths et al. (127) are:

Fundamental criterion

- The biomarker predicts the later development of disease.

Technical criteria

- The biomarker should detect a major part or at least a fixed percentage of total oxidative damage to the target molecule *in vivo*.
- It must employ validated measurement technology and is measurable within the limits of detection (LOD) of the method.
- The coefficient of variation between different assays of the same sample should be very small in comparison with the differences between subjects or the effect of experimental manipulations (e.g. antioxidant supplementation) upon a subject.
- It should be free of confounding factors from dietary intake.
- It should ideally be stable on storage, not being lost, or formed artefactually, in stored samples.
- For human use, it is preferable if it can be measured in easily obtainable samples, e.g. blood, urine, saliva, skin biopsy.

Validation of biomarkers requires two steps. The fundamental validation showing that the changes in the biomarker reflect the development of the disease. The analytical validation includes development of methods, elimination of methodological artefacts, analysis of reference materials and quality control (13).

Markers of oxidative stress *in vivo* can be classified into three major groups: markers of oxidative damage to lipids, proteins and DNA.

Lipids

The lipid rich sites *in vivo* are the lipid-carrying lipoproteins and cellular membranes. Lipids can be oxidized, halogenated or nitrated by different reactive species apart from hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$) and superoxide anion ($O_2^{\bullet -}$) (13). Lipid peroxidation is thought to proceed by radical mediated abstraction of a hydrogen atom from a methylene carbon on a polyunsaturated fatty acid (PUFA) or a PUFA side chain (128). It is a complex process and a wide range of products are formed in variable amounts (128). Lipid peroxidation plays a significant pathological role especially in atherosclerosis (8). The extent of lipid peroxidation can be determined by measuring the losses of PUFAs, the amounts of primary peroxidation products like hydroperoxides, conjugated-dienes, isoprostanes and the amounts of secondary products, such as hydrocarbon gases (13;127).

Isoprostanes

The best available biomarker of lipid peroxidation appears to be the isoprostanes (IPs). They are prostaglandin-like compounds formed from PUFA with at least three double bonds such as linolenic acid and arachidonic acid (produce F_2-IPs); eicosapentaenoic acid (produce F_3-IPs) and docosahexaenoic acid (produce F_4-IPs). Most of the work is done on F_2-IPs and their metabolites. The most abundant F_2-IP is 8-isoprostagladin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$). They are measured by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) or immunoassays, the latter being less reliable. GC-MS techniques are quite established but the sample preparation techniques are quite tedious (129). LC-MS techniques do not require derivatization step and are less prone to artifacts (129). F_2-IPs can be measured in plasma, urine and other body fluids with urine generally considered as a better matrix due to following reasons. The correct storage of plasma and tissues samples is important as artefactual lipid oxidation and IP formation can occur unless antioxidants like butylated hydroxytoluene (BHT) are added (13). In plasma and tissues most IPs are esterified with phospholipids and it is important to identify between free and total IPs (13). Another disadvantage in plasma is that it is not possible to measure them over a period of time due to their short half-life

(approximately 18 minutes) in plasma (127). These challenges can be overcome by measuring IPs in urine. However, local kidney peroxidation could be a problem for urinary F_2-IP measurement. This can be overcome by measuring both 8-iso-$PGF_{2\alpha}$ and its metabolite 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in urine (13;130). Other factors like fasted samples can increase the F_2-IPs levels in urine and thus these samples are to be regarded with caution (13). Additionally, 24-hour urine sample collection and standardization of F_2-IPs concentrations with creatinine are suggested (9).

Increased levels of IPs in plasma / urine are observed in animals and humans associated with oxidative stress, including renal, cardiovascular, lung, neurodegenerative diseases, diabetes, hypertension (120;131) . Modulation of IPs by antioxidant supplementation is also reported (127;132-134). Different PUFAs generate different IPs, giving a possibility to follow the peroxidation of individual PUFAs *in vivo*.

Aldehydes

Many aldehydes and carbonyls are generated during lipid peroxidation, including malondialdehyde (MDA) and 4-hydroxynonenal. The concentrations of free aldehydes *in vivo* are probably low, because they are readily conjugated to proteins. Spectrophotometric, chromatographic and antibodies based methods are used (13;127). The most commonly measured aldehyde in plasma or urine is MDA, usually measured by thiobarbituric acid (TBA) test. MDA is heated with TBA under acidic conditions, forming an adduct which is detected spectrophotometrically at 532 nm (127). The test is highly unspecific as many others compounds not related to lipid peroxidation reacts with TBA forming the similar chromogen (13;127). Measuring the fluorescence of MDA-TBA adduct instead will improve the specificity. However, the assay becomes even more specific by performing a chromatographic separation of MDA-TBA from interfering chromogens (127). Use of MDA as a biomarker for lipid peroxidation has a number of limitations as reviewed elsewhere (13;127). One of the important limitation include that the formation of MDA could be due to other

processes and not always due to lipid peroxidation. The test is, however, still widely used due to the low cost and easy to perform

Peroxides

Lipid peroxidation also generates different peroxides and a number of different assays measuring total and individual peroxides are available (13). Some of the assays available are iodine liberation, ferrous oxidation xylenol orange (FOX), cyclooxygenase (COX), derivatives of reactive oxygen species (d-ROMs). d-ROMs are the indirect way of measuring hydroperoxides in plasma/serum. The hydroperoxides are decomposed in presence of metal ions forming $RO^{\bullet}$ and $RO_2^{\bullet}$ radicals. These radicals oxidize the chromgen N,N,-diethylparaphenylendiamine, thereby changing its colour from pink to red, which is measured spectrophometrically (135). The hydroperoxides in biological samples also arise from the attack of reactive species on other organic molecules. Thus, d-ROMs are not a specific biomarker of lipid peroxidation but a more general biomarker for oxidative stress. Hayashi et al. (136) have measured d-ROMs in serum of smokers and non-smokers and found that the smokers have higher levels of plasma d-ROMs.

Conjugated dienes

The oxidation of PUFAs form conjugated dienes that absorb ultraviolet (UV) light in the 230-235 nm wavelength range (13). Measurement of dienes is useful in pure lipids detecting an early stage lipid peroxidation. However, an application of this technique to human body fluids or their extracts results in an overestimation due to presence of other substances that also absorb strongly in the same UV range. Although extraction of lipids before their measurement could solve this problem, the dienes can also arise from other sources besides lipid peroxidation such as diet or microbial metabolism in the gut (13). Thus, the validity of this biomarker for lipid peroxidation is questionable.

Breath analysis

Exhaled air contains F_2-IPs, aldehydes and a range of hydrocarbons including ethane and pentane (120). Ethane is derived from n-3 PUFAs and pentane from n-6 PUFAs. Both gases can be measured by GC. The expired gas is passed through an absorbent at low temperatures to bind and concentrate the hydrocarbons, which are desorbed and measured (13). Hydrocarbons are however minor end products of peroxidation and their formation are affected by the transition metal ions to decompose peroxides, O_2 concentrations and altered liver metabolism (13;127). Other disadvantages of this biomarker are that other sources including presence of bacteria and environmental factors like air contamination due to motor vehicles can contribute to hydrocarbons (13;127). Contamination of pentane due to the presence of another hydrocarbon isoprene can give erroneous results (13;127). This can be solved by measuring ethane instead of pentane. Exhaled hydrocarbons are also difficult to measure routinely in large human studies, requiring cumbersome equipments. Increased exhalation with age, hyperoxia, smoking, scleroderma and some lung diseases has been demonstrated in some human studies (9;13).

DNA

Oxidative DNA damage seems to relate to an increased risk of cancer development with age. At molecular level, DNA damage can take many forms, ranging from specifically oxidized purine and pyrimidine bases (more than 20 such oxidative lesions have been identified) to gross DNA changes such as strand breaks, sister chromatid exchange and the formation of micronuclei (137;138). The DNA oxidation products formed depend upon the type of reactive species involved, its rate of production and the ability of the cell to repair the damage (137). The oxidative DNA damage can be measured as steady state damage in accessible cells like leukocytes, colonic endothelium and buccal cells (137). The steady state damage reflects the balance between damage and repair, and an increase in oxidative DNA damage could be due to increased damage and/or decreased repair. Another approach is to measure the total *in vivo* oxidative DNA damage by measuring products of DNA repair that are excreted in urine (13).

Oxidized nucleosides/bases

Among the most commonly measured biomarkers of total oxidative DNA damage is the modified nucleoside 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxo-dGuo) or base 8-oxo-7,8-dihydroguanine (8-oxo-dGua) , considered to be a biologically important lesion. The mutagenic and carcinogenic potential of any modified DNA base is reflected in its miscoding properties. The presence of 8-oxo-dGuo residues in DNA can lead to GC to TA transversion, and if not repaired can lead to mutagenesis and may be carcinogensis (127). Other less studied oxidized bases like 2-hydroxy-adenine, 8-hydroxy-adenine and 5-OH-Cyt have also shown to be mutagenic (127).

The techniques for the measurement of 8-oxo-dGuo in cells and urine are usually chromatographic employing liquid chromatography-electrochemical detection (LC-ED) or GC-MS or liquid chromatography-tandem mass spectrometry (LC-MS-MS), comet assay and immunoassay (127;139). The results from different techniques vary over a range of three orders of magnitude in cells and the European standards Committee on Oxidative DNA Damage (ESCODD, 1997) has been testing ability of different laboratories using different method to measure 8-oxo-dGuo in standard samples, calf thymus DNA, pig liver, oligonucleotides, HeLa cells and in lymphocytes isolated from blood of volunteers (139).

Chromatographic

The chromatographic based techniques measure 8-oxo-dGuo in both cells and urine. In cells, LC-ED is capable of measuring 8-oxo-dGuo with high accuracy but there is a lack of consensus on basal levels measured (127;139). Advantages with GC-MS are that many different modified purines and pyrimidines can be detected but GC-MS failed to detect a dose response of induced 8-oxo-dGuo and cannot reliably measure low levels of damage (127;139). New techniques like LC-MS-MS, although quite specific has yet not proved capable of measuring low levels of oxidative DNA damage. The limitations with chromatographic methods include artefactual oxidation during sample preparation, low limits of detection and lack of specificity (especially in HPLC-ED) (127). The artefactual oxidation can be avoided by use of antioxidants,

eliminating traces of oxygen, room temperature derivatisation, and use of repair endonucleases to liberate base products instead of acid hydrolysis (127). It is also important to accurately measure unoxidized base (dGuo) as the concentration of 8-oxo DGuo is expressed relative to dGuo.

Besides measuring in cells, urinary 8-oxo-dGua and 8-oxo-dGuo can be used to assess oxidative DNA damage. It is assumed that the products of oxidative DNA damage are excreted in urine and represents the primary product of repair *in vivo*. The advantage of analysing in urine is that the levels of 8-oxo-dGuo and 8-oxo-dGua do not depend upon the diet in humans and are not susceptible to oxidation (127). GC with isotope dilution MS and LC-MS-MS are the techniques that are currently being used for their analysis (140). The limitation of this biomarker is its specificity i.e., processes other than repair processes can also contribute to 8-oxo-dGua and 8-oxo-dGuo levels in urine (127).

Comet assay

An alternative approach to measure DNA damage in cells is by comet assay. This technique can be applied to any type of cells provided they have been isolated from tissue without degradation (127). In human studies, lymphocytes are used as surrogate tissues. Comet assay is a simple, fast and sensitive technique that measures both DNA strand breaks and oxidized bases (127). The DNA strand breaks are measured by subjecting the isolated nucleoids from lysed cells to alkaline electrophoresis. The negatively charged DNA are attracted to the anode but only the loops of DNA possessing a break are free to migrate, presenting the image of a comet with a tail (127) called as comet images. The quantitation of these images is done by computer image analysis or a visual scoring system (class 0 no tail, class 4 all DNA in the tail). The calibration is achieved against X-ray irradiated cells, where the frequency of strand breaks introduced is known. The measurement of oxidized bases, however, requires an additional step of DNA incubation with bacterial repair endonucleases. The endonucleases recognise and remove damaged bases and make nicks at the resulting abasic sites in the DNA (127). Endonuclease III detects

oxidized pyrimidines and formamidopyrimidine DNA glycosylase (FPG) recognises altered purines including 8-oxo-dGuo (127). These repair enzymes (endonuclease III or FPG) are added to isolated nucleiods and parallel gels (with and without enzymes) are run. The comet scores are subtracted, calculating the degree of oxidative damage.

Advantage of this assay as compared to chromatographic techniques is that there is little chance for artefactual oxidation. The potential problems with assay can be calibration, linearity, lesions occurring in close proximity with in one DNA loop will be registered as one lesion leading to underestimation or unspecificity of FPG leading to overestimation (127).

Immunoassay

The enzyme-linked immunoabsorbent assays (ELISA) have also been used to assess 8-oxo-dGuo. Both polyclonal and monoclonal antibodies are commercially available (140). These assays are much simpler, more reproducible, robust and versatile in comparison to chromatographic methods. The baseline values in healthy individuals are also consistent between different laboratories (120;127). Since DNA extraction and hydrolysis are required prior to ELISA, the artefactual oxidation should be avoided. On comparing chromatographic methods and ELISA a poor correlation is observed, additionally the latter giving two to four times higher values than the former (127;140). The main reason is the cross-reactivity of antibodies employed in ELISA towards other species in urine (127;140).

Proteins

Oxidative protein damage is studied to lesser extent as compared to lipid peroxidation and DNA oxidation. It could be due to the complexity since 20 different amino acids can be attacked by reactive species forming different oxidative products (127). Free radical attack on proteins can generate amino-acid radicals, which may crosslink or react with O_2 to give peroxyl radicals ($RO_2^{\bullet}$) resulting in a variety of reactive species (120). Of the 20 amino acids, aromatic and sulphydryl containing residues have considered to be most susceptible to oxidation. Markers of protein oxidation include

protein carbonyls derivatives, oxidized amino acid side chains, protein fragments, and formation of advanced glycation end products (AGEs) (137).

Protein carbonyls

Protein carbonyls are formed by the oxidative cleavage of the peptide main chain or by oxidation of the following amino acid side chains: arginine, lysine, praline and threonine (137). Additionally some lipid peroxidation products including HNE and MDA can form adduct with amino acids generating carbonyls (137). The techniques employed to measure carbonyls are immunodetection by ELISA, western blot and chromatographic (127). Proteins carbonyls are first derivatized by 2,4-dinitro hydrazine (2,4-DNPH) to yield the corresponding 2,4-dinitrophenyl hydrazones (127). Hydrazones can be separated by HPLC and detected by UV or immunochemically by commercially available antibodies.

Protein carbonyls are a generic and not specific marker of protein oxidation. Since carbonyls are usually formed by oxidative mechanisms, and this assessment of protein modification can provide a reasonable index of oxidative stress index. Protein carbonyls have been most strongly linked with aging and in Alzheimer diseases (137).

Other biomarkers from the amino acid oxidation products include kynurenines (from tryptophan), bityrosines, valine and leucine hydroxides, L-dihydroxyphenylalanine (l-DOPA), ortho-tyrosine, 3-nitro tyrosine (attack of $ONOO^-$ upon proteins) (127).

Although studied to lesser extent, the oxidative damage to proteins could be important as the changes in the proteins can alter the function of receptors, enzymes and can cause secondary damage to other biomolecules like inactivation of DNA repair enzymes (120).

Oxidative stress biomarkers- conclusion

Oxidative biomarkers for measuring oxidative damage are in the establishing phase and the results are showing that some are better than others. The final conclusions

about biomarkers of lipids, proteins and DNA oxidation) are taken from the ESSCOD recommendation (127) that are as follows:

- For lipid oxidation biomarkers, determination of lipid peroxides and IPs is considered suitable with further work necessary on urinary IP metabolites and hydrocarbon gases. Measurement of TBARS, MDA and conjugated dienes is considered inappropriate.

- For DNA oxidation biomarker, measuring 8-oxoGua in DNA is suitable, but further work is required on other oxidized base analysis, enzyme sites in the comet assay and 8-oxo-dGuo measurement in urine by chromatography or ELISA. Strand break analysis alone is inappropriate.

- Protein oxidation biomarkers are in earlier stages of validation. The suitable candidates, however, include nitrated amino acids, protein-bound tyrosine oxidation products, protein-bound tryptophan products, and methionine sulphoxide.

1.3.2 Decrement in antioxidant defence

Another approach is to measure the decrement in the antioxidant defence as increased production of free radicals will result in the more utilisation of various endogenous- and dietary- antioxidants. Some of these are described below.

Glutathione

It is well accepted that cellular concentrations of GSH and its oxidized form-GSSG are related to oxidative stress, and that the level of GSH and GSSG reflect the degree of oxidative stress in different pathophysiological conditions (25;30;141).

Measurements of total GSH, free GSH and GSSG have been used extensively to estimate oxidative stress or the redox environment of cells. Many researchers have estimated oxidative stress by using the ratio [GSH]/[GSSG]. This is convenient for many measurements since the absolute concentrations are not needed. Recently, the

redox state of the GSSG/2GSH couple calculated with the Nernst equation was suggested to describe the redox environment of biological fluids, cell organelles, cells, or tissue (141). Redox state is considered to be better parameter as compared to the ratio since it takes into account the correct stiochiometry involving GSH and GSSG, which is a two-electron process. The redox (reduction) potential of this couple calculated by Nernst equation given by:

$$GSSG + 2H^+ + 2e^- \longrightarrow 2GS$$

$$E_{hc} = -240 - (59.1/2) \log ([GSH]^2/[GSSG]) \text{ mV}$$

There are many redox couples in a cell that work together to maintain the redox environment, but Schafer and Buettner (141) suggest that the redox state of the GSSG/2GSH couple can serve as an important indicator of redox environment since it is the most abundant redox couple in the cell. Additionally, decrease in GSH redox potential has been observed with aging, smoking, and diabetes (142;143)..

GSH can be measured in whole blood, erythrocyte, plasma, leukocytes, platelets and various tissues. The total concentration of GSH in whole blood and serum/plasma is about 2 mM and 5 μM, respectively. Erythrocytes constitute 40-50% of the blood volume and contribute about 99 % of GSH in whole blood. Whole blood analysis of GSH therefore almost exclusively reflects erythrocyte GSH.

The ratio of GSH and oxidized forms of GSH is different in erythrocytes as compared to plasma (144;145). In erythrocytes, the major form of GSH is the reduced free form of GSH, which accounts for about 76 % of the total GSH. GSSG represents 8 % and PSSG accounts for about 16 % (145). In plasma, the reduced free from of GSH represents about 62 % of the total, whereas GSSG and mixed disulfides represent 18 % and protein-bound represents about 20 % (144). Thus, the redox state of GSH is more oxidized in plasma as compared to the erythrocytes (146).

Both chromatographic and enzymatic methods are available for the measurement of GSH and GSSG (147;148). The reported concentrations of total GSH (sum of free

GSH, GSSG, mixed GSH disulphides and protein bound GSH), free GSH and GSSG in blood, erythrocytes, leukocytes, platelets and plasma vary considerably. The reasons for this variation are as follows:

- GSH can be easily oxidized during sample preparation
- GGT which is found in plasma and most cells can degrade GSH if the enzyme is not inhibited during sample preparation
- Plasma values are easily increased by incidental and non-reproducible leakage from erythrocytes having 1000-times higher GSH and GSSG concentration than plasma.

The challenges for the determination of GSH and GSSG in blood and erythrocytes have been discussed elsewhere (149-152). The precautions for the determination of GSH and GSSG in plasma are discussed by Jones et al. and Sakhi et al. (153;154).

A major conclusion of these articles is that it is essential to inhibit GGT mediated degradation of GSH by either serine borate or acivine, and to stop artificial oxidation of GSH by blocking the thiol group of GSH before the precipitation of proteins with an acid.

A number of different sample preparation and analytical methods are available, and a variety of GSH forms may be measured. The nomenclature that has been used to describe these different forms of GSH is inconsistent. For example, total GSH may in some texts refer to the sum of free reduced GSH and GSSG, while in other texts it reflects the sum of all forms of GSH (i.e. also including PSSG and GSH bound to other small molecular weight thiols). Thus, it is important to define which GSH form is being measured.

Total antioxidant capacity

Antioxidants form an intricate network in the protection against reactive species and analysing only one antioxidant may give an in complete overview. This provides the

rationale for analysing all the antioxidants present in the sample by measuring total antioxidant capacity (TAC). TAC is usually measured in plasma /serum. The main determinants of the plasma /serum TAC are urate (60%), ascorbate and protein thiols (155).

Several different assays are available to measure TAC. Assays involving oxidants that are not necessarily pro-oxidants are ferric ion reducing antioxidant power (FRAP), Trolox equivalence antioxidant capacity (TEAC) and cyclic voltammetry whereas assays involving oxidants that are necessarily pro-oxidants are total radical trapping antioxidant parameter (TRAP), oxygen radical absorbance capacity (ORAC) (156).

Anticoagulant and storage of samples are critical factors that affect the TAC. This is due to ascorbate, which is among the major contributors to TAC and decays rapidly on storage. The use of TAC as an indicator of the antioxidant defence has been contradicting due to its increases under the enhanced oxidative stress conditions (127). This is mainly due to high contribution of uric acid in plasma, obscuring the changes in the concentrations of other antioxidants and thus limiting its usefulness.

Measurement of dietary antioxidants- carotenoids, vitamin E and ascorbic acid

Plasma levels of various carotenoids, vitamin E and ascorbic acid are decreased in smokers and various oxidative stress related diseases as compared to healthy controls, and thus can be used as biomarkers for oxidative stress. However, their plasma/serum levels are affected by diet, and thus it is important to have a control over changes in dietary patterns during the measurement period.

Carotenoids

Carotenoids are usually measured in plasma/serum. HPLC with ultraviolet (UV) detection is the most common method employed for their determination (157). Carotenoids are highly lipophilic and require organic solvents like isopropanol, hexane for their extraction from the plasma samples.Carotenoids are light sensitive

and must be protected during sample preparation. The type of anticoagulant used in blood sampling tubes also effect the concentration of carotenoids. The plasma concentration of total carotenoids is approximately 1-3 µmol/L (84).

Non-invasive method for measuring carotenoids in skin and eye using Raman spectroscopy are also developed (158). The method is shown to be precise, specific, sensitive and well suitable for clinical as well as field studies (159). The only disadvantage with this method is that only total, and not individual, carotenoid concentration can be measured.

Vitamin E

Vitamin E isomers are usually measured in plasma/serum. Vitamin E exhibit native fluorescence and thus HPLC with fluorescence detection is the commonly employed technique for their determination. For biological samples, reversed phase chromatography is employed and the separation of all the vitamin E isomers is achieved by only some (160;161) but not all columns. Protection from light and type of anticoagulant in blood sampling tubes should be considered during sample preparation. EDTA plasma is considered to be most suitable for their determination (162). The concentration in plasma is 15-40 µmol/L: Being lipid soluble, it is more accurate to express vitamin E concentration as the molar ratio of vitamin E/cholesterol (163).

Ascorbic acid

Ascorbic acid is oxidized to DHAA and the ratio of DHAA/total ascorbic acid can be used as a biomarker for oxidative stress. Ascorbic acid and DHAA are usually measured in plasma, using HPLC with UV detection. Ascorbic acid can be easily oxidized at neutral or alkaline pH and thus acidification of plasma should be done immediately (164). The type of anticoagulant also effects its concentration and heparin plasma is most suitable for its determination (164). The concentration of ascorbic acid in plasma is approximately 30-80 µmol/L.

1.4 Cancer and oxidative stress

It is evident that elevated levels of ROS/RNS can lead to cancer (10). The specific mechanism by which oxidative stress contributes to the development of carcinogenesis is not fully understood. The most common explanation, however, is the DNA oxidative damage by ROS/RNS. Cancer development is a micro-evolutionary and multistage process and requires the cumulative action of multiple events that occur in one cell alone (165). These events include a three stage model: a permanent change in one somatic cell genetic material (initiation); the expansion of the mutated cell alone (promotion); and the malignant conversion into cancer (progression) (165). ROS/RNS can stimulate carcinogenesis by acting at all three stages.

Initiation

Initiation involves a non-lethal mutation in DNA producing an altered cell. Oxidative DNA damage occurs through the attack of ROS/RNS especially $OH^{\bullet}$ (10). The yield of the individual DNA modifications is highly dependent on which reactive species are involved (166). Thus, $^1O_2$ induces prefentially 8-oxodGuo whereas $OH^{\bullet}$ attack upon DNA generates a whole series of DNA damage by a variety of mechanisms (165;166). These include sugar and base modifications, strand breaks and DNA-protein cross-links. Modified DNA bases (pyrimidine and purine) are one of the most common lesions and 8-oxodGuo represents one of the most studied lesions due to its mutagenic properties. Some RNS deaminate DNA bases to mutagenic lesions such as $ONOO^-$ forms 8-nitroguanine (8-NG) in DNA. 8-NG rapidly detaches from the DNA, leaving potentially mutagenic apurinic sites (10). The damage caused by ROS/RNS can still be repaired by interrupting temporarily their cell cycle stage G1, S or G2 (check points), repairing the damage, and resume division (167). However, a very small part of the oxidative DNA lesions escape repair and represents an important mutagenic potential that accumulates with age (168). Higher doses of ROS/RNS increase the chance that the DNA lesions may not be effectively encountered by DNA repair. Thus, exposure of mammalian cells to various reactive species increases

mutagenesis. The contribution of reactive species to carcinogenesis becomes even more important if DNA base changes occur in certain oncogenes and tumour suppressor genes.

Promotion

The promotion stage is characterised by the clonal expansion of mutated cells by the induction of cell proliferation and /or inhibition of programmed cell death (apoptosis) (167). This stage is still reversible and the cells proceeding towards progression requires optimal production of ROS and continuous presence of tumour promotion stimulus (167). A high level of oxidative stress is cytotoxic to the cell and halts proliferation by inducing apoptosis. A low level of oxidative stress can in fact stimulate the cell division in the promotion stage and thus stimulate the promotion of tumour growth (167).

Progression

Progression is the third and irreversible stage of the carcinogenesis process, leading to the transition of the cell from benign to malignant (167). This stage involves accelerated cell growth, tissue invasion, increased genetic instability and metastasis (165).

ROS can increase proliferation in tumour cells by decreasing gap juctional communication (GJC), thus, corrupting the contact growth inhibition signals (10). ROS is also involved in another important step in tumour progression, angiogenesis. Angiogenesis generates new blood supplies that feed the malignant cells as required for the growth of any tumour (167). The ROS involvement include generation of reactive oxygen intermediates by cancer cells, damage to vascular basement membranes mediated by endothelial injury or perturbation, and direct activation of latent matrix metalloproteinases (165). The experimental tumours have also increased levels of inducible nitric oxide synthase (iNOS) and the nitric oxide ($NO^{\bullet}$) released increase vascular permeability enhancing tumour progression and angiogenesis (165).

1.5 Oxidative stress and head and neck squamous cell carcinoma

The head and neck cancer include cancers of the oral cavity (lip, the base of tongue, gum, floor of mouth and palate) pharynx (oropharynx, hypopharynx and nasopharynx) and larynx (Figure 4) (169).

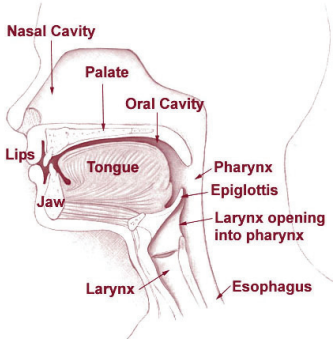


Figure 4 Different regions of head and neck cancer. The figure is taken with permission from the following site: http://training.seer.cancer.gov/ss_module06_head_neck/unit02_sec02_anatomy.html

Ninety percent of these cancers are squamous cell carcinomas (91). Worldwide, head and neck cancers are the seventh most common cancers (91), with approximately 540 000 new cases annually and 271 000 deaths (170). In Norway 400-500 new cases are registered each year (171). Despite advances in treatment, five-year survival rates has improved marginally and are around 50-60 % over the past 20 years (91). While the primary cancers have been successfully treated, the development of second primary tumours (SPT), relatively high median age of 50 years or more and co-morbidities are the important factors determining survival in these patients (91;172). The incidence of this cancer is about three times more in men as compared to women and more in African-American people than in white people (91;173). HNSCC has strong link to oxidative damage as major risk factors include tobacco use and alcohol consumption in developed countries and betel quid chewing and bidi smoking in many developing countries (91;174;175). Other factors include dietary habits, papillomavirus infection and polymorphisms of GSH S-transferase (91;174;176-178).

The geographic area with the highest incidence from oral and pharyngeal cancers are south-central Asia, Europe, Oceania and southern Africa, while for laryngeal cancer are South America, south-central and western Asia, and southern, central and western Europe (91).

1.5.1 Smoking and alcohol

Smoking and alcohol are the established risk factors and contributes to about 90 % in the development of HNSCC cancer (91). While smoking rates are declining in the developed world, they are increasing in the developing world. Striking variations in head and neck cancer sites and incidence seen among different regions, cultures, and demographic groups are due in large part to differentiating patterns of tobacco and alcohol abuse (174;179). Alcohol is an important independent promoter of carcinogenesis and is a contributive factor in at least 75 % of HNSCC cases (173). Alcohol appears to have an effect on risk of HNSCC independent of tobacco smoking, but these effects are consistently significant only at the highest level of alcohol consumption (173). It also appears that alcohol consumption potentiates the carcinogenic effect of tobacco at every level of tobacco use (173).

Smoking and oxidative stress

Cigarette smoke contains approximately 10^{17} oxidant molecules per puff (180). The free radicals in cigarette smoke are produced (a) during the burning of tobacco and the smoking process and (b) generated when the gas phase and the constituents from the particulate matter are oxidized in the smoke aerosol or dissolved in oxygenated aqueous solutions or biological media (181). Analysis of both particulate phase, gas-phase in cigarette smoke have detected different oxidants like superoxide anion ($O_2^{\bullet -}$), quinones, hydrogen peroxide (H_2O_2), redox active metals (iron and copper), other heavy metals (cadmium, lead, mercury and arsenic), nitric oxide ($NO^{\bullet}$) and different nitroso carbon-centered radicals (181).

Most of these species are highly reactive and increase oxidative damage in smokers. Free radicals from cigarette smoke have shown to cause peroxidation of the PUFA in cell membranes (182). Isoprostanes are increased in exhaled air, plasma and urine in several studies (180;182). A dose-response relationship was observed between the number of cigarettes and urinary 8-iso-$PGF_{2\alpha}$ (180). Increased levels of MDA have been found associated with current smoking status in population based studies (180). Biomarkers for protein oxidation, 3-nitrotyrosine is elevated in plasma and platelets of chronic smokers (183;184). The acute effects of smoking (an effect measured during the 24 hours after smoke exposure) increases markers of oxidative stress in humans, animals and in-vitro models as reviewed by van der Vaart et al. (182) The exposure to oxidant chemicals in smoke are also associated with depletion of endogenous levels of antioxidants. Cigarette smoking decreases plasma total antioxidant capacity, serum levels of ascorbic acid, α-tocopherol, different carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lutein/zeaxanthin) (180;185;186). Diet also influences the levels of these antioxidants. After correcting for dietary intake, plasma ascorbic acid and β-carotene still showed an inverse relationship with cigarette consumption (180;186). Plasma levels of GSH and cysteine decreased in smokers as compared to non-smokers (187). Serum selenium and erythrocyte GPx activities were also lower in smokers (185).

Thus, smoking increases oxidative damage and stress in biological systems.

Alcohol and oxidative stress

The toxicity of alcohol abuse is mediated through high intake of ethanol. The metabolic pathways of ethanol could produce free radicals that affect the antioxidant system (188). Ethanol ingestion causes an increase in free radical generation in the liver by induction of microsomal cytochrome p-450, conversion of xanthine dehydrogenase into xanthine oxidase in cytosol and increases one electron reduction in mitichondria increasing levels of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (188).

The ESR in combination with spin trapping method has revealed that ethanol oxidation results mainly (more than 80%) in the formation of a free radical identified as 1-hydroxylethyl radical (HER) (189). HER have shown to have a high reactivity towards ascorbic acid, GSH, α-tocopherol and DNA (Nakao and Augusto et al 1998, Stoyanovsky et al 1998). The elevation of oxidized LDL, advanced glyoxidation end products and acetaldehyde protein adducts have been observed in heavy alcoholic drinkers (188). A decrease level of GSH in liver has been observed both in ethanol fed animals as well as patients with alcoholism (188;190) . Lower levels of α-tocopherol in plasma and liver have been observed in humans and rodents after chronic alcohol intake, and the lowering of the α-tocopherol is inversely correlated with the detection of lipid peroxidation markers (190). The enteral alcohol model has shown a marked decline in enzymatic activity and protein concentrations of various enzymes involved in antioxidant defence (190).

Thus, alcohol abuse also increases oxidative stress.

There is a strong association between heavy alcohol use and cigarette smoking. More than 80% of alcohol dependent patients are reported to smoke cigarettes (191;192). In head and neck cancer patients, the combined effect of high alcohol consumption in current smokers is more than additive indicating a positive synergy (91;173).

1.5.2 Diet and HNSCC

Although smoking and drinking are the major risk factors for developing HNSCC, diet rich in fruits and vegetables have also shown to play an important role. The role of diet on the development and risk of HNSCC has been studied extensively. Recently the report from WCRF (91) have clearly indicated that non-starchy vegetables, fruits and food rich in carotenoids probably protect against mouth, pharynx, larynx and lung cancer. Meta-analysis and several review articles also conclude that the consumption of fruit and vegetables is associated with a reduced risk of oral and pharyngeal cancer (64;65;193). The preventive effect of fruits and

vegetables is also seen in smokers, drinkers and those with both smoking and drinking habits (194). Other dietary factors apart from fruits and vegetables like meat, saturated fat have shown to increase the risk of HNSCC but the results are still inconclusive (91).

The mechanisms behind the protective effect of fruits and vegetables are not fully understood as described earlier. Probable mechanisms are their antioxidant role, modulation of carcinogenic metabolism, affecting cell transformation and differentiation, inhibition of cell proliferation and oncogene expression.

2. Study aims

This work is part of a project where the overall goal is to explore which biomarkers of antioxidant defence and oxidative stress are associated with survival in HNSCC patients; and can diet rich in fruits and vegetables, modulating these biomarkers, improve survival in these patients. This study particularly has focussed on the low molecular weight plasma endogenous and dietary antioxidants.

The specific aims are

- to develop and evaluate improved sensitive chromatographic techniques for measuring plasma GSH and GSSG
- to establish which food groups contribute to the total antioxidant intake in Norwegian healthy individuals and to study whether intake of dietary antioxidants correlates with low molecular weight plasma antioxidants.
- to study which low molecular weight plasma antioxidants are affected by radiotherapy and their association with survival in HNSCC patients

3. Summary of papers

Paper I: Simultaneous quantification of reduced and oxidized glutathione in plasma using a two dimensional chromatographic system with parallel porous graphitized carbon columns coupled with fluorescence and coulometric electrochemical detection

In Paper I, a fully validated, accurate and precise chromatographic method for the simultaneous quantification of GSH and GSSG in human plasma was developed. The developed method avoids artificial oxidation and degradation of GSH during sample preparation. In order to achieve this, we have used the commercially available Stabilyte tubes to generate plasma from blood. The special feature of these tubes is that the anticoagulant is acidic citrate resulting in a final plasma pH of 5.3, thereby stabilizing GSH and avoiding oxidation of GSH during plasma formation. After blood collection, serine borate buffer was immediately added to inhibit GGT. The buffer also contains bathophenanthroline disulphonate (BPDS) to chelate metal ions that otherwise can catalyze oxidation of GSH. In addition, we block the very reactive –SH group of GSH within 2 min after plasma generation with monobromobimane (MBB) to stop unwanted formation of disulphides. GSH bound to MBB, GSMBB, was detected with a fluorescence detector (FLD). GSSG was detected with an electrochemical detector (ED) optimized to provide lowest possible limits of detection (LOD). By combining both FLD and ED via column switching, we were able to detect both GSH and GSSG in a single run. The derivatized samples were stable up to 8 months in -80°C. A Hypercarb^{TM} column that does not require ion pair reagent for the retention of GSSG under reversed phase conditions was used. The column is stable in the pH range of 1-14 such that 100 µl of strong acidic supernatant could be injected without any column deterioration.

Paper II: Simultaneous and robust trace analysis of reduced and oxidized glutathione in minute plasma samples using dual mode fluorescence detection and column switching HPLC

In this work we have further developed the method presented in paper I, increasing both sensitivity and selectivity such that GSH and GSSG can be analyzed in 50 µL human plasma. In cases where blood/plasma volume is quite limited, the assay can detect GSH and GSSG in 5 µL plasma if 80% of the sample is injected into the HPLC system. The factors causing the oxidation and degradation of GSH during sample preparation have been taken care of and were similar as described in paper I. The plasma was generated in Stabilyte tubes and the –SH group of GSH was blocked with MBB within 2 min of plasma generation. GSH was detected as a MBB derivative by FLD. In this method, the GSSG was detected by FLD after on-line postcolumn derivatization with *ortho*-phthalaldehyde (OPA) at a pH of 12.4. The important factors involved in derivatization, namely coil volume, OPA concentration in mobile phase 2 and coil temperature were optimized to obtain lowest possible LOD for GSSG. The sensitivity for GSSG was improved by a factor of 20 as compared to method in paper I. The method is fully validated, performs well, is very robust for endogenous plasma concentrations of GSSG, and is currently in use for determination of GSH, GSSG and its redox potential in different clinical studies.

Paper III: Intakes of antioxidants in coffee, wine, and vegetables are correlated with plasma carotenoids in humans

The objective of this study was to determine the contribution of various food groups to total antioxidant intake, and to assess the correlations of the total antioxidant intake from various food groups with plasma antioxidants. We collected 7-d weighed dietary records in a group of 61 adults with corresponding plasma samples, and used data from a nationwide survey of 2672 Norwegian adults based on an extensive food frequency questionnaire (FFQ). No plasma samples were collected in the nationwide survey. The total intake of antioxidants was approximately 17 mmol/d with β-carotene, α-tocopherol, and ascorbic acid contributing <10%. The intake of coffee contributed approximately 11.1 mmol or 64%, followed by fruits and berries (1.8 mmol), tea (1.4 mmol), wine (0.8 mmol), cereals (i.e., all grain containing foods; 0.8 mmol), and vegetables (0.4 mmol). The strongest correlations between intake of

antioxidants from various food groups and plasma antioxidants were for carotenoids; tocopherols and thiols showed much lower correlations. The intake of total antioxidants was significantly correlated with lutein, zeaxanthin, and lycopene, whereas noncoffee antioxidant intake was significantly correlated with plasma lutein and β-carotene. Intakes of coffee, wine and vegetables were significantly correlated with zeaxanthin, β-carotene and α-carotene respectively.

Paper IV: Post-radiotherapy plasma total glutathione is associated to outcome in patients with head and neck squamous cell carcinoma

In paper IV, four plasma thiols, namely GSH, cysteine, homocysteine and cysteinyl glycine, were measured in healthy controls (n= 51) and head and neck squamous cell carcinoma (HNSCC) patients (n= 29). The method employed in present study reduces disulphides prior to derivatization and protein precipitation. Thus, the total thiols measured in present study included free reduced thiols (TSH), disulphides (TSST), mixed disulphides (TSSX) and protein bound disulphides (TSSP). Patients received external beam radiation to a dose of 60-70 Gy for 5-7 weeks. The blood samples were taken immediately after the end of radiotherapy. Among thiols, total GSH was significantly lower and total homocysteine was significantly higher in patients as compared to controls. The differences were, however, not significant after adjusting for gender, smoking and BMI. The 29 HNSCC patients were followed for 36 months after the end of radiotherapy and 14 patients died during this period. Among patients, post-radiotherapy plasma total GSH was significantly higher in survivors than in non-survivors. There were no significant differences between the levels of other thiols (total cysteine, homocysteine or cysteinyl-glycine) in these two groups. Plasma total GSH was also associated to survival in these patients, and the patients with lowest total GSH levels had the lowest overall survival.

Paper V: Post-radiotherapy plasma lutein, α-carotene and β-carotene are positively associated with survival in patients with head and neck squamous cell carcinoma.

The aim of our study was to compare plasma dietary antioxidants, namely tocopherols and carotenoids (i.e. biomarkers of dietary intake of fruits and vegetables), in 29 HNSCC patients with 51 healthy controls, and to explore the possibility that these antioxidants may be related to survival among HNSCC patients. Patients received external beam radiation to a dose of 60-70 Gy for 5-7 weeks. The blood samples were taken immediately after the end of radiotherapy. We observed that among dietary antioxidants, post-radiotherapy plasma carotenoids (lutein, zeaxanthin, α-carotene, β-carotene, lycopene and total) were lower in HNSCC patients than controls. Among the patients, 18 died and 11 were still alive during median follow-up of 55 months for survivors. The survival analysis showed significant positive association of plasma carotenoids (lutein, α-carotene and β-carotene) with both overall and progression free survival in HNSCC patients. We found no significant differences between plasma tocpherols in patients as compared to controls. Further, none of the tocopherols were associated to survival in patients. This may indicate that increased intake of fruits and vegetables, reflected by plasma carotenoids, may reduce risk of premature death or recurrence of tumor in these patients.

Paper VI: Pre-radiotherapy plasma carotenoids and markers of oxidative stress are associated with survival in head and neck squamous cell carcinoma patients

The aim of this study was to compare plasma levels of antioxidants and markers of oxidative stress in HNSCC patients with healthy controls, the effect of radiotherapy on these biomarkers and their association to survival in HNSCC patients. Seventy eight HNSCC patients were included in this study. Follow-up samples at the end of radiotherapy were obtained in 60 patients. The control group comprised of 100 healthy individuals matched with respect to age range, gender and smoking with HNSCC patients. Antioxidants, both endogenous and dietary, and oxidative stress markers were measured in plasma samples of controls and patients. Dietary antioxidants included 6 carotenoids (lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene), 4 tocopherols (α-, β-, γ- and δ-tocopherol) and ascorbic

acid. The endogenous antioxidants measured were GSH redox potential, total GSH and cysteine. In addition, total antioxidant capacity was measured by FRAP and FRAP without uric acid. Among oxidative stress parameters, d-ROMs, GGT, ratio oxidized/total ascorbic acid were measured.

All dietary antioxidants (carotenoids, tocopherols and ascorbic acid), total antioxidant capacity (FRAP, FRAP without uric acid) and total cysteine were significantly lower in HNSCC patients as compared to controls and dietary antioxidants decreased during radiotherapy. Among oxidative stress biomarkers, d-ROMs were significantly higher than controls and increased during radiotherapy. During the median follow-up time of 28 months for survivors, 23 patients died due to HNSCC, one patient died of lung cancer and one patient died of unknown cause. Among dietary antioxidants, pre-radiotherapy plasma levels of carotenoids (lutein, β-cryptoxanthin, β-carotene and total), were positively associated to progression free- and overall- survival. No significant associations were observed between pre-radiotherapy plasma levels of other biomarkers with survival. However, the patients with a high relative decrease in plasma levels of FRAP and a high relative increase in plasma levels of d-ROMs during radiotherapy had a higher survival rate.

In conclusion, we observed that patients with high pre-radiotherapy plasma carotenoids (i.e., biomarkers of fruits and vegetables) are associated with a prolonged survival. Further, increase in oxidative stress during treatment also increases survival in these patients. Thus, the therapeutic potential of optimizing antioxidant status and oxidative stress by increasing intake of fruits and vegetables should be explored further.

4. General discussion

We have described the average daily intake of antioxidants in the Norwegian healthy individuals. It was found that coffee was the major contributor (64-68 %) to the daily antioxidant intake in Norwegian diet. The intake of total antioxidants from different food groups was significantly correlated with plasma carotenoids. Other plasma antioxidants like tocopherols and thiols showed much lower correlations. These plasma antioxidants (carotenoids, tocopherols and thiols) were also studied in head and neck squamous cell carcinoma (HNSCC) patients, both before and after the end of radiotherapy. Among antioxidant biomarkers, several dietary antioxidants were found to be significantly lower as compared to healthy controls. HNSCC patients receive high dose of radiation therapy. The usual therapeutic radiation dose schedule includes 2 Gy/day, 10 Gy/week, for a total of 50-70 Gy in 6 weeks. The plasma dietary antioxidants decreased and oxidative stress biomarkers increased significantly after the radiotherapy in these patients. Among plasma dietary antioxidants, carotenoids, both before and after radiotherapy, were found to be significantly and positively associated with survival. Among endogenous antioxidants, post-radiotherapy plasma total GSH was significantly and positively associated with survival. Relative decreases in total antioxidant capacity FRAP and relative increases in oxidative stress biomarker d-ROMs during radiotherapy were also significantly associated with survival.

4.1 Methods for assessment of antioxidant and oxidative stress status

There are no single biomarkers available that can define the complete antioxidant and oxidative stress status in humans. Thus, a number of biomarkers are measured in different sample matrixes to study antioxidant and oxidative stress status both in healthy individuals and patients. The feasibility in a clinical study and accuracy in the measurement of the chosen biomarkers are important factors. The purpose/aim and

the study population in the clinical trials are also deciding factors in choosing the biomarker. Our aim was to study antioxidant intake, correlation of antioxidant intake to *in vivo* antioxidants, effect of radiotherapy on *in vivo* antioxidants and association of *in vivo* antioxidants to survival in HNSCC patients. Thus, we have chosen biomarkers that could enlighten these aspects in healthy individuals and patients. All the biomarkers were measured in plasma as the status of biomarkers in this biological fluid could both reflect the oxidative stress in less accessible tissues and systemic oxidative stress in the whole body. Further, the plasma levels of dietary antioxidants have shown to be correlated to the intakes of fruits and vegetables (195;196). The antioxidants status was studied by measuring both endogenous and dietary antioxidants. Among endogenous biomarkers, methods were developed for the determination of plasma reduced GSH, GSSG, total GSH and total cysteine. The dietary antioxidants are studied by measuring 6 different carotenoids, 4 different tocopherols and ascorbic acid. Total antioxidant capacity was measured by FRAP and FRAP after removing uric acid. The oxidative stress was studied by measuring d-ROMs, GGT and ratio oxidized/total ascorbic acid. Not all biomarkers were measured in all the clinical studies as some of the methods for the measurement of biomarkers were not available and were developed simultaneously.

4.1.1 Endogenous antioxidants- Glutathione method development

Reduced and oxidized GSH

Among endogenous antioxidants, GSH is considered to be one of the major nonprotein thiol involved in the antioxidant cellular defence. This tripepetide is involved enzymatically in reduction of hydroperoxides and nonenzymatically to maintain vitamin E and ascorbic acid in reduced and functional forms (197). In doing so GSH is oxidized to GSSG, which is either reduced enzymatically by GR or excreted from cells into extracellular fluids. During severe oxidative stress and detoxification reactions involving GSH, the concentration of GSH may decrease and the concentration of GSSG may increase in the affected cells. This results in a

decreased export of GSH and an increased export of GSSG to plasma, thereby altering the GSH redox state of the plasma pool. Thus, the altered plasma GSH and GSSG concentrations can, thus, reflect GSH/GSSG status and oxidative stress in other less accessible tissues (197). Measurement of GSH/GSSG in plasma is not straightforward as a number of factors can affect the accuracy in its measurement.

Sample handling

The factors affecting the accuracy in the measurement of GSH include mainly the sample handling as shown in paper I. GSH, being an antioxidant, is easily oxidized during the centrifugation of whole blood in order to get the plasma. This was clearly observed when we used different anticoagulant tubes for the preparation of plasma from the whole blood. It was observed that pH of the blood sampling tube was important in stopping the oxidation of GSH. The lower the pH the lesser was the oxidation. Stabilyte tubes have shown to preserve the ratio of GSH to GSSG as compared to tubes containing EDTA, heparin and citrate anticoagulants. The anticoagulant used in Stablilyte tubes is acidic citrate such that the pH of the plasma prepared from these tubes was 5.3 whereas the pH of the other tubes was 7.4. One could suspect that there could be the leakage of GSH from erythrocytes in Stabilyte as compared to other tubes as erythrocytes contain 1000 times more GSH than plasma. Any leakage of GSH from erythrocytes, either as reduced or oxidized form, will increase the total GSH level in plasma as the total GSH measured by the our method measures all the forms of GSH after reducing them to GSH. The total GSH was similar in all the tubes and we concluded that the high levels of reduced GSH in the plasma of Stabilyte were not due to the leakage from erythrocytes. Similar results for reduced GSH have been observed by Williams et al. (198). GSSG concentration was significantly higher in other anticoagulant tubes as compared to Stabilyte but still could not account for the lower GSH concentrations. The reason could be that the GSH makes disulphides with cysteine and proteins that we do not measure with this method (199-202).

Another factor that could affect GSH concentration is the enzyme GGT. GGT cleaves GSH at the γ-glutamyl bond into amino acid glutamate and dipepetide cysteinyl glycine, thereby reducing its concentration. Andersson et al. (203) have shown that total GSH was 53 % lower in plasma when GGT was not inhibited. After blood collection, serine borate buffer was immediately added to inhibit GGT. The inhibition is produced by formation of serine-borate complex which binds at the γ-glutamyl binding site of the light subunit of GGT (204). The serine borate buffer used also contained BPDS to chelate metal ions that otherwise can catalyze oxidation of GSH (147;148;200).

Thus, the Stabilyte tubes were chosen for the measurement of reduced, oxidized and total GSH in plasma in the clinical study presented in paper VI. The clinical studies presented in the paper III and IV, however, did not use Stabilyte tubes as the method was not completely developed. We have, thus, measured only total GSH in first clinical study with HNSCC as total GSH is much more stable in different anticoagulants as shown in paper I.

The blood taken in these tubes could be stored up to 40 min at room temperature without any significant increase in the GSH levels.

Detection

After the plasma preparation, the –SH group of GSH was still prone to oxidation as the acidification of samples with different acids slows GSH oxidation but it does not prevent it completely (149;205). Thus, it was important to block the –SH group of GSH in plasma before storage. Since GSH does not contain any native spectrophotometric or fluorescence properties we have blocked the –SH group of GSH with monobromobimane (MBB). MBB not only blocks the –SH group of GSH but it imparts it with the fluorescence properties such that GSH was detected by a fluorescence detector with high sensitivity. GSSG also has very poor and non-specific spectrophotometric or fluorescence properties and the concentration of GSSG in plasma is in the nmol/L range. GSSG could be determined either indirectly by subtraction method or directly by electrochemical detector (ED) or mass

spectrometry (MS) detector. For the indirect method, the GSSG is reduced either chemically or enzymatically to GSH. The reduced GSH is then measured by reacting it with thiol specific fluorescence probe. The plasma contains different forms of bound GSH apart from GSSG, which are also reduced in this process thereby reporting higher GSSG concentrations. The concentration of GSSG in plasma is in nmol/L and after reduction the increase in reduced GSH is very less which is difficult to measure with accuracy. Among the direct methods, GSSG could be measured by ED and MS detector. The limit of detection with both detectors is in the pmol range as shown in Table 2 in paper II. The ED used in paper I contains an array of 8 electrochemical cells. The advantage of such a series of cells was that increasing potential could be applied to the cells, which will oxidize the impurities in the sample matrix and increase the specificity for GSSG. The availability and the price of the detector were also deciding factors in choice of the detector. We have, thus, chosen ED for detection of GSSG in paper I.

After analyzing plasma samples for GSH and GSSG in other clinical studies, we were able to see the limitations with the developed method. The limitations were frequent changing of the precolumn/analytical column 1 due to the large injection volume in order to reach the required sensitivity for the detection of GSSG. The second limitation was the specificity of GSSG in some plasma samples. GSSG being an oxidized product requires high electrochemical potential in order to be further oxidized. At high potentials, many other compounds in the plasma could be oxidized. The series of 8 electrochemical cells used for the detection of GSSG oxidized most of the interferences. However, in some plasma samples all the impurities were not removed and interfered with the GSSG detection. We have, thus, developed the existing method and improved both the sensitivity and specificity for GSSG such that injection volume was reduced to 10 µL (paper II). The ED was replaced by an on-line postcolumn reactor and a fluorescence detector (FLD). The fluorescence probe used in the detection of GSSG was *ortho*-phthalaldehyde (OPA). OPA is a thiol specific fluorescence probe and GSSG usually does not react with these reagents due to lack of free –SH group. Cohn and Lyle (206) reported for the first time that GSSG reacted

with OPA at a very high pH, namely 12. Hissin and Hilf et al. (207) confirmed the results and developed a method for the measurement of GSH and GSSG in a test tube. The method suffered with the contamination from other organic compounds in the sample as OPA at pH 12 reacted with other compounds besides GSSG. It was then suggested to perform a chromatographic separation of GSSG prior to reaction with OPA (208). The relative fluorescence intensities of other amino acids like glutamic acid, cysteine, cystine, leucine and glycine were less than 1% as compared to GSSG thereby showing high specificity of OPA for GSSG at pH 12 (207) . After replacing ED with on-line postcolumn reactor and a FLD, several important postcolumn reaction parameters, namely coil volume, concentration of OPA in mobile phase and temperature for postcolumn reactor were optimized to provide lowest possible LOD for GSSG (paper II). The sensitivity for GSSG was, thus, improved by a factor of 20 as compared to method in paper I and we were able to measure GSH and GSSG simultaneously in 50 µL or less plasma volume. The current method was, thus, used for the analysis of plasma GSH and GSSG in paper VI.

Total Glutathione

GSH exists in different forms in plasma and blood. The different forms are following: reduced GSH, GSH bound to other thiols like cysteine, oxidized form GSSG, GSH bound to protein. The distribution of these forms is different in blood and plasma as shown by Mills et al. (145) and Mansoor et al. (144). The method for the total GSH used in the clinical studies (papers III, IV and VI) includes the reduction of all the bounded forms of GSH to the reduced form prior to derivatization and protein precipitation. A homocysteine kit was used for the determination of GSH in plasma. The commercially available homocysteine kit is validated only for the determination of plasma homocysteine but could also be used for the determination of total GSH in plasma. Thus, the kit was further validated for the total GSH determination in plasma. Different anticoagulants were also tested for the sample collection for total GSH. Results have shown that Stabilyte, heparin and EDTA sampling tubes could be used for total GSH.

4.1.2 Dietary antioxidants

The methods for the determination of dietary antioxidants, namely carotenoids (lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene) and tocopherols (α-, β-, γ- and δ-tocopherol) were developed and validated in our laboratory. Different anticoagulants were tested and the results are as follows: For carotenoids, EDTA, heparin and serum anticoagulants can be used (data not shown). For tocopherols, serum and EDTA anticoagulants are appropriate anticoagulants (162). The blood samples from controls and patients in papers IV, V and VI were collected in different anticoagulants tubes. The control values were, thus, corrected for the appropriate dilution factor due to different volumes of anticoagulants and eventual effect of anticoagulants on these biomarkers before statistical analyses. The ascorbic acid method was developed by Karlsen et al. (209) and the blood sample preparation and analysis was done accordingly as described. FRAP was measured as described by Benzie et al. (155) and Halvorsen et al. (210).

4.1.3 Oxidative stress parameters

The oxidative stress was measured by measuring increase in the d-ROMs, GGT and ratio of oxidized/total ascorbic acid. The blood samples for d-ROMs, GGT, oxidized ascorbic acid (DHAA) and total ascorbic acid were taken and analyzed that avoided any artefact formation.

The biomarkers of DNA, proteins and lipids oxidation were not measured due to unavailability of these analytical methods in our laboratory. A large variation in the values of these biomarkers has been observed depending upon the method employed for measurement (127). Additionally, the type of biomarkers that are representative for studying oxidation of DNA, proteins and lipids were not fully established at the time of initiation of these studies, such that appropriate sample handling was not accomplished either.

4.2 Sources of antioxidants in healthy Norwegian individuals

Among healthy Norwegian individuals, the main dietary contribution to total antioxidant intake is coffee (Paper III). These results were observed in 61 individuals with 7-day weighed dietary record and were consistent with a much larger nationwide survey including 2672 Norwegian adults. The diets in 2672 Norwegian adults were characterized using an extensive, self-administered FFQ. The mean daily intake of coffee was ca 480 mL and accounted for 64-68 % in both the studies. Although it is reported that coffee contains high amount of antioxidants but such a high contribution of coffee to antioxidant intake in Norwegian population is not observed before. Similar results were observed in a study done by Pulido et al. (211) where coffee contributed to 66 % to antioxidant intake in Spanish population. Pellegrini et al. (212) have also shown that coffee and tea were the major contributor to antioxidant intake in Italian women.

Coffee contains several different antioxidants such as caffeine, polyphenols including chlorogenic acid (an ester of caffeic acid and quinic acid), volatile aroma compounds and various heterocyclic compounds (213). Both caffeic acid and chlorogenic acid are absorbed in humans, and plasma antioxidants increase after coffee intake (214-216). Epidemiological studies and meta-analysis have also shown that coffee is associated with reduced risk of type 2 diabetes, Parkinson disease, cirrhosis, liver cancer (217;218). The results showing association between coffee intake and risk or mortality due to CVD have not been conclusive (213;217;219). The effect of coffee intake in CVD is dependent upon the dose (U-shaped association with coffee intake) and type of coffee (filtered, boiled) consumed (213;217;219). In conclusion, a moderate intake of 3-5 cups/day filtered coffee could be protective.

After coffee, fruits and berries were the next contributor to total antioxidant intake. Fruits and berries are rich in different polyphenols, carotenoids, vitamins and minerals. Intakes of most common dietary antioxidants, namely β-carotene, α-

tocopherol and ascorbic acid are also shown to be correlated to antioxidant intake from fruits and vegetables (paper III).

The correlation of total antioxidant intake from different food groups and plasma antioxidants was studied. Among the plasma antioxidants measured, only carotenoids (lutein, zeaxanthin, α-carotene, β-carotene and lycopene) showed significant correlations. As these 5 carotenoids are only minor contributors to the total intake of antioxidants; other dietary antioxidants may theoretically save, recharge or salvage these carotenoids when they have been used in a redox reaction in accordance with the hypothesis that many antioxidants may interact in a network (95;220). Valtuena et al. (221) have also shown that plasma β-carotene is more strongly associated to intakes of ascorbic acid and E rather than β-carotene intake itself. The authors suggested that it may reflect either sharing of dietary sources (primarily ascorbic acid) and/or a protection of plasma β-carotene by other antioxidants. The other plasma antioxidants like tocopherols and thiols showed much lower correlations with total antioxidant intake from different food groups. These observations might suggest that the plasma tocopherols and thiols are not in complete equilibrium with plasma carotenoids, which could be due to different chemical reactivities or compartmentalization of the plasma pools. Besides, our study included healthy individuals having relatively low systemic oxidative stress. Stronger correlations and antioxidant networking including tocopherols and thiols might be more important and, thus, be observed in clinical situations with prolonged oxidative stress.

Thus, in Norwegian healthy individuals the main contributors to antioxidant intake are coffee, fruits and berries. Among different low molecular weight plasma antioxidants, carotenoids have shown strongest correlations with total antioxidant intake from different food groups.

4.3 Plasma antioxidants in head and neck cancer patients

The assessment of plasma antioxidants in HNSCC patients were studied in two clinical studies 1 and 2. The first was a pilot study with 29 HNSCC patients and 51 healthy controls. The patients received external beam radiotherapy to a total dose of 60-70 Gy during the treatment period of 5-7 weeks (papers IV and V). The main purpose of this study was to investigate how the plasma antioxidants status of the patients after the end of radiotherapy and being cured of cancer was associated to survival. Hence, post-radiotherapy blood sample were taken at two different time points, immediately after the end of radiotherapy and 6-weeks after the end of radiotherapy. The levels were compared to healthy controls. In the clinical study 2 (paper VI), we wanted to find out how radiotherapy affects the plasma antioxidant levels and how pre-radiotherapy plasma antioxidant status is related to survival in these patients. This information is quite important since it is speculated that the adverse effects of radiotherapy could be reduced by giving antioxidants to the patients during the radiotherapy. The purpose of radiotherapy is to kill the cancer by increasing the amount of free radicals. The antioxidant supplementation during radiation therapy poses a conundrum for the radiation oncologist, as antioxidants that protect normal cells from reactive oxygen species may provide the same benefits to cancer cells and reduce the efficacy of treatment (222;223). Thus, it is quite important to know what actually happens to plasma endogenous and dietary antioxidants before and after radiotherapy and how these antioxidants are related to survival in these patients. Hence, in the second clinical study 78 patients were included and blood samples were taken both before and after the end of the radiotherapy. Patients in this study also received a total dose of 50-70 Gy for 5-7 weeks.

The populations in both the studies were quite similar with respect to the type of cancer, age and radiation therapy dose. However, the study presented in paper VI had more men and non-smokers; more patients received assisted enteral nutrition and were operated before radiotherapy as shown in Table 4. The assisted nutrition received by patients in the clinical study 2 was also fortified with β-carotene (130

μg/100 mL) whereas the assisted nutrition given to patients in the first clinical study did not contain any carotenoids.

Table 4 Different basic parameters in clinical studies 1 and 2. In the first clinical study, only post-radiotherapy samples were available whereas in the second clinical study both pre- and post- radiotherapy samples were taken.

Variables		**Study 1 (paper IV and V)**	**Study 2 (paper VI)**	**Study 2 (paper VI)**
		Post-radiotherapy	**Post-radiotherapy**	**Pre-radiotherapy**
		n = 29	**n = 60**	**n = 78**
Age (years)*		64 (43-90)	62 (37-85)	63 (34-85)
BMI*		24 (17-31)	25 (16-40)	25 (16-40)
Gender	Male	22 (76%)	56 (93%)	69 (88%)
	Female	7	4	9
Smoking status	Non-smoker	6 (23%)	25 (42%)	28 (36%)
	Smoker	20	35	50
Disease stage	Stage 1 and 2	7 (24%)	20 (33%)	26 (33%)
	Stage 3 and 4	22	40	52
Treatment	Surgery before radiotherapy	6 (21%)	22 (37%)	30 (39%)
	Radiotherapy	23	38	48
Nutrition form	Assisted nutrition	8 (28%)	24 (40%)	24 (40%)
	Oral diet	21	36	36

* Values are presented as median (range)

4.3.1 Dietary antioxidants

Plasma levels, effect of radiotherapy and survival

The levels of pre- and post- radiotherapy plasma dietary antioxidants in HNSCC patients were compared to healthy controls (papers V and VI). The levels of several pre-radiotherapy dietary antioxidants including carotenoids were significantly lower in HNSCC patients than matched controls as shown in paper VI. This could be due to low intake of fruits and vegetables and other life style factors including smoking. Reports have also shown that low intakes of fruits and vegetables (91;193), low dietary intakes of carotenoids (194;224) and lower pre-diagnostic plasma β-carotene levels (225;226) are associated with increased risk of developing HNSCC. Post-radiotherapy antioxidants were also significantly lower than controls as shown in paper V. Since the blood samples in paper V were taken at the end of radiotherapy, lower levels of the antioxidants in patients could be due to other additional factors such as (1) eating problems due to the adverse effects of radiotherapy and (2) utilization of antioxidants during radiotherapy. After correcting either for various confounding factors such as BMI (representing food intake and physical activity), smoking and age or using matched controls, the results were still significant for carotenoids. This indicates that the food intake did not account for the observed difference. A low intake of fruits and vegetables both before and during the radiotherapy seems to be the most plausible explanation for the lower carotenoid plasma levels in patients. Steward et al. (227) have also shown that the patients that have been treated for early-stage oral cavity carcinoma and are free of cancer have a much lower mean daily intake of fruits and vegetables and dietary antioxidants than matched controls.

In paper VI, we have further studied the effect of radiotherapy on these antioxidants. During radiotherapy we observed a significant decline in plasma levels of carotenoids, tocopherols and ascorbic acid. Among carotenoids, β-carotene showed a non-significant decrease ($p = 0.4$). In paper VI, 24 patients received assisted enteral nutrition fortified with β-carotene (130 μg/ml). Subgroup analysis in patients without

assisted enteral nutrition revealed a borderline decline also for β-carotene ($p = 0.06$). It has also been shown that the half-lives of serum carotenoids during depletion (intake of low carotenoid diet) are 4-11 weeks (228). Thus, the decrease in plasma carotenoids during radiotherapy (a period of 5-7 weeks) could be due to low intake of fruits and vegetables during treatment period and utilization of these antioxidants during that period.

The survival was studied with both post-radiotherapy (paper V) and pre-radiotherapy plasma antioxidant levels (paper VI). Our results showed that among dietary antioxidants, 3 post-radiotherapy plasma carotenoids (lutein, α-carotene and β-carotene) were significantly and positively associated with survival in these patients after correcting for BMI (paper V). None of the tocopherols were associated to survival. These 3 carotenoids were also found to be most reliable for assessing changes in fruit and vegetable intakes (229-235). Paper III has also shown that antioxidant intake in different food groups is correlated to plasma carotenoids and not tocopherols in healthy individuals. Since the patients have lower intake of fruits and vegetables as reflected through their plasma carotenoid levels and data shown elsewhere (227;236), we suggest that increasing the intake of this food group may reduce risk of premature death or recurrence of tumor in HNSCC patients.

In paper VI, we have explored how the pre-radiotherapy plasma levels are associated to survival. Results have shown that plasma carotenoids (lutein, β-carotene and β-cryptoxanthin and total) are significantly and positively associated to survival. Other dietary antioxidants such as tocopherols and ascorbic acid have shown no significant associations with the survival. Recent report from WCRF has also concluded that foods containing carotenoids probably protect against mouth, pharynx, larynx and lung cancer (91). In elderly subjects the plasma carotene concentration was associated with a lower overall mortality risk (70). This risk was also observed for both cancer and cardiovascular mortality. Unlike our study, Mayne et al. (237) has shown that among carotenoids, only plasma lycopene is associated to overall survival in early stage head and neck cancer patients. The study population was, however, different

from our study as one of the inclusion criteria in the study was that the patients had completed their treatment and considered free of cancer at any site. Hence, many deaths related to treatment toxicity and other causes were not recorded.

In conclusion, plasma levels of both pre- and post- radiotherapy carotenoids (biomarkers of fruits and vegetables) in HNSCC patients are lower than healthy controls, decrease during radiotherapy and have shown positive associations with overall and progression free survival.

4.3.2 Endogenous antioxidants

Plasma levels, effect of radiotherapy and survival

Among endogenous antioxidants, we measured plasma GSH in papers IV and VI. In paper IV the method for the determination of both reduced and oxidized was not available and thus we have only measured plasma total GSH. Both in papers IV and V, the total GSH was not significantly lower in patients as compared to healthy controls. The post-radiotherapy plasma levels of total GSH measured in HNSCC patients in paper VI (mean total GSH = 5.63 μM) were, however, higher than those in paper IV (mean total GSH = 3.68 μM) showing that the patients in the paper VI have a better endogenous antioxidant status. In both papers, same method for the analysis of total GSH is employed. The reason for better plasma GSH status of patients in the clinical study 2 could be due to following reasons. In the clinical study 2 there were fewer smokers, fewer advanced stage (III and IV) patients and more patients received assisted nutrition as compared to study 1. Additionally, more patients in clinical study 2 were operated before radiotherapy as compared to clinical study 1. The postoperative radiotherapy has shown to have a significant positive association with both progression free- and overall- survival, log rank $p = 0.03$ and 0.005, respectively. Thus, patients in clinical study 2 were better followed up as compared to patients in study 1. Another reason for high total GSH concentration in paper VI could be that plasma in clinical study 2 was collected in Stabilyte tubes and serine borate buffer that inhibits the enzyme gamma glutamyl transpeptidase (GGT) was

added. The enzyme GGT cleaves GSH thereby reducing its concentration in samples. Serine borate binds GGT thereby inhibiting its activity.

The effect of radiotherapy on plasma GSH redox potential and total GSH was studied in paper VI. Although dietary antioxidants decreased significantly after radiotherapy, we observed no decrease in endogenous antioxidants. Similar results are shown by Mukndan et al. (238) and Bhuvarahamurthy et al. (239) where they have studied plasma reduced GSH before and after radiation therapy in uterine cervix cancer patients. However, Jadhav et al. (240) have shown decreased levels of plasma GSH+GSSG after one fraction of radiation therapy in cervix cancer. This difference could be due to the fact that they measured GSH+GSSG only after one fraction of radiation therapy and not after completing the whole treatment.

The association of plasma GSH levels with survival is studied in papers IV and VI. Paper IV has shown that the patients with post-radiotherapy plasma total GSH levels over median has longest overall survival rate. The pre-radiotherapy GSH redox potential and total GSH have shown no association with survival in paper VI.

4.3.3 Total antioxidant capacity (TAC)

Plasma levels, effect of radiotherapy and survival

Plasma TAC was measured by FRAP and FRAP without uric acid (paper VI). Uric acid accounts for about 60 % antioxidant capacity in FRAP (155). The roles of uric acid as an antioxidant is still inconclusive and thus we have measured both FRAP and FRAP without uric acid. Both FRAP and FRAP without uric acid were significantly lower in HNSCC patients than healthy controls. However, none of them decreased significantly during radiotherapy. Similar effect of radiotherapy on TAC measured by TRAP was shown by Erhola et al. (241) in lung cancer patients. To our knowledge no data is available showing effect of radiotherapy on TAC in HNSCC patients. Pre-radiotherapy plasma TAC was not associated with survival. However, relative decreases in FRAP showed a positive association with survival. The effect could be

due to decrease in uric acid levels as FRAP without uric acid showed a borderline association with survival (HR 0.53, 95 % CI 0.23-1.21, p = 0.13).

4.3.4 Oxidative stress biomarkers

Plasma levels, effect of radiotherapy and survival

The oxidative stress biomarkers measured in plasma were d-ROMs, GGT and ratio oxidized/total ascorbic acid (paper VI). d-ROMs were significantly higher in patients as compared to controls and increased significantly during radiotherapy also. Another marker of oxidative stress GGT also increased significantly during radiotherapy. We observed no significant association between pre-radiotherapy plasma levels of markers of oxidative stress and survival. However, a high relative increase in d-ROMs during radiotherapy was significantly and positively associated with survival in patients. These results indicate that the patients that are responding to radiotherapy and increase oxidative stress during treatment have a prolonged survival. Thus, care must be taken if antioxidant administration is considered during the treatment period.

4.4 Future perspectives

We measured dietary antioxidants with different chemical properties in order to study their association with antioxidant intake in healthy subjects and with survival in HNSCC patients. The results show that plasma carotenoids were significantly associated to total antioxidant intake from different food groups in healthy individuals and were positively associated to survival in HNSCC patients. Among endogenous antioxidants, we have studied only glutathione and found no strong associations. Thus, other endogenous antioxidants like different enzymes involved in antioxidant defence like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase could be studied in this population.

Since plasma carotenoids (biomarkers of fruits and vegetables) have shown strongest effects in our studies, intervention trials with increasing fruits and vegetables intake

in these patients at an appropriate timing and studying survival could be conducted. This group of patients suffer from a lot of eating problems and thus intervention in the form of juice or soups could be suggested. Nutritional counselling, especially during the treatment period will motivate the patients to maintain their fruit and vegetable intake and thus should be integrated in the regular treatment schedule.

5. Conclusions

- A sensitive and specific chromatographic method for the simultaneous determination of GSH and GSSG in plasma using dual mode fluorescence detection has been developed. Use of Stabilyte blood sampling tubes for plasma preparation, inhibition of GGT and blocking the –SH group of GSH with monobromobimane, are important factors for maintaining GSH and GSSG ratio in plasma. The specificity and sensitivity of this assay allows analysis after fingertip sampling, blood sampling from infants or multiple blood sampling from experimental animals without sacrificing the animal.

- Coffee intake, followed by fruits and berries are the major contributors to antioxidant intake in healthy Norwegian individuals. Several plasma carotenoids are correlated to total antioxidant intake (FRAP) by different food groups (coffee, tea, wine, cereal, fruits and vegetables).

- In HNSCC patients, both pre- and post- radiotherapy plasma carotenoids were significantly lower in patients compared to healthy controls. All plasma dietary antioxidants (carotenoids, tocopherols and ascorbic acid) decreased significantly during radiotherapy. No effect of radiotherapy was observed on endogenous antioxidants. Oxidative stress, measured by d-ROMs and GGT increased significantly during radiotherapy. Among the different antioxidants measured, pre-radiotherapy plasma carotenoids have shown strongest positive association with survival in these patients. Additionally, the relative increases in plasma levels of d-ROMs and relative decreases in plasma levels of total antioxidant capacity (FRAP) during radiotherapy were also associated with a prolonged survival in these patients.

- Our data show that high levels of both pre- and post- radiotherapy plasma carotenoids are beneficial for survival in HNSCC patients. Plasma carotenoids are biomarkers of fruits and vegetable intake. This may suggest that HNSCC

patients should be advised to increase their intake of fruits and vegetables rich in carotenoids, both before and after radiotherapy. However, since a reduced risk of disease relapse was observed in individuals experiencing a greater degree of oxidative stress during treatment period, our data may suggest that administration of antioxidants should not be combined with radiotherapy. The effects of carotenoid-rich fruits and vegetables before, during and after the radiotherapy on survival in HNSCC patients should be studied in future intervention trials.

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